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PRINCIPAL INVESTIGATOR: Santosh R. D'Mello, Ph.D.

CONTRACTING ORGANIZATION: University of Texas at Dallas
Richardson, TX 75083-0688

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Santosh R. D'Mello, Ph.D.

E-Mail: dmello@utdallas.edu

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14. ABSTRACT:

Apoptosis is a cell-suicide process that is required for the normal development of the nervous system, but that can be aberrantly activated in neurodegenerative diseases and following exposure to neurotoxins. The intracellular pathways by which these different physiological and pathophysiological stmuli cause neuronal death has not been characterized. In our original application we proposed the hypothesis that certain components of the signaling pathways activated by different apoptotic stimuli might be shared. four of these molecules had been implicated in the regulation of neuronal survival. In a request for supplemental funding that was submitted in 2001, we had proposed to extend our investigation to another well-studied apoptosis-regulatory molecule - the serine-threonine kinase, Akt. In 2002, we submitted a statement of modification of work as part of our annual report. In 2003, we submitted another request for supplemental funding. The funds were to be used to study a novel and exciting chemical compound called GW6074, which we found had neuroprotective properties. Finally, in 2004 we were provided with yet another supplement to studu the mechanism of action of GW5074 and examine whether this compound could prevent neurodegeneration and improve behavioral outcome in an in an animal model of Parkinson's disease.

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INTRODUCTION:

Apoptosis is a cell-suicide process that is required for the normal development of the nervous system. Aberrant and inappropriately regulated apoptosis can, however, lead to undesirable neuronal loss such as that seen in certain neurodegenerative diseases. Apoptosis can also be induced in various neuronal populations by chemical and biological neurotoxins. The intracellular pathways by which these different physiological and pathophysiological stmuli cause neuronal death has not been characterized. *In our original application we proposed the hypothesis that certain components of the signaling pathways activated by different apoptotic stimuli might be shared.* Our expectation was that once identified, such molecules could serve as ideal targets for the development of approaches to protect or treat individuals against the actions of neurotoxic agents. We proposed to focus our attention specifically on four signaling molecules- NF-κB, p38 MAP kinase, caspases, and mGluR4. All four of these molecules had been implicated in the regulation of neuronal survival. The primary model for studying these molecules was cultures of cerebellar granule neurons (CGNs). These neurons undergo apoptotic death when switched from medium containing depolarizing medium (high potassium medium or HK) to non-depolarizing medium (low potassium medium or LK). LK-induced neuronal death can be prevented (and survival maintained) by treatment with agents such as cyclic AMP, IGF-1, or lithium.

In a request for supplemental funding that was submitted in 2001, we had proposed to *extend our investigation to another well-studied apoptosis-regulatory molecule - the serine-threonine kinase, Akt.* In 2002, we submitted a statement of modification of work as part of our annual report. In 2003, we submitted another request for supplemental funding. The funds were to be used *to study a novel and exciting chemical compound called GW6074, which we found had neuroprotective properties.* Finally, in 2004 we were provided with yet another supplement to studu the mechanism of action of GW5074 and examine *whether this compound could prevent neurodegeneration and improve behavioral outcome in an in an animal model of Parkinson's disease.*

BODY

We have made considerable headway on a number of different fronts and our studies have resulted in 12 publications and two reviews. For the purpose of better presentation, we describe our findings under the following sub-headings – (1) NF-κB and the regulation of neuronal survival, (2) IκB-β phosphorylation and neuronal survival, (3) Akt as a potential convergent point in survival-promotion signaling, (4) Relationship between NF-κB and Akt, (5) Analysis of GW5074 as a neuroprotective compound, and (6) 3' substituted indolone as a scaffold for the development of neuroprotective drugs.

1. NF-κB and the regulation of neuronal survival

We found that pharmacological inhibition of NF-κB blocks CGN survival in HK. Although no change in expression or subcellular localization of the various NF-κB or IκB proteins can be detected when CGNs are switched from HK to LK medium, we found that the DNA-binding and transcriptional activity of NF-κB is nevertheless reduced Phosphorylation of p65, which stimulates its activity in some cases, is also unchanged. Overexpression of p65 inhibits LK-induced apoptosis whereas overexpression of IκB-α (which would inhibit NF-κB) promotes apoptosis even in HK-medium. We proposed to investigate the mechanism by which NF-□B activity was regulated during neuronal apoptosis. We found that p65

interacts with the transcriptional coactivator, CREB-binding protein (CBP) in healthy neurons. LK-induced decrease in NF-κB activity is accompanied by a reduction in the interaction between p65 and CBP. While the expression of CBP is unchanged in neurons primed to die, the reduced association with p65 in LK is accompanied by hyperphosporylation of CBP. These alterations occur within 6h of LK treatment, a time around which these neurons become committed to death. We found that the hyperphosphorylation of CBP in LK can be mimicked by inhibitors of protein phosphatase 2A (PP2A) and PP2A-like phosphatases suggesting that LK-induced increase in CBP phosphorylation may result from an inactivation of PP2A phosphatases. Treatment with these PP2A inhibitors also causes a reduction in p65-CBP association. While PP2A, PP4, and PP6 are all expressed inCGNs, only PP6 was found to interact with CBP implicating it as the phosphatase relevant to the regulation of CBP hyperphosphorylation. In addition to stimulating CBP hyperphosphorylation, treatment with PP2A-like phosphatase inhibitors induces cell-death.

To gain insight into the mechanism by which LK-induced CBP phosphorylation occurs, we initiated efforts to identify the region within the protein in which the apoptosis-associated hyperphosphorylation occurs. We generated a number of GST-CBP constructs and used these in *in vitro* kinase assays with lysates from HK or LK-treated neuronal cultures. These analyses mapped the LK-regulated site to a region spanning amino acids1662 - 1840 of CBP. This region contains a consensus sequence for phosphorylation by cyclin-dependent kinases (CDKs). Consistent with the involvement of CDKs, we have recently found that LK-induced hyperphosphorylation of CBP is inhibited by roscovitine, a pharmacological inhibitor of several CDKs. Several other kinase inhibitors did not block hyperphosphorylation of CBP. As published by other groups and by our own lab, inhibition of CDKs with pharmacological inhibitors or using dominant-negative CDK constructs, blocks LK-induced neuronal apoptosis. Taken together, our results are consistent with the possibility that LK-induced apoptosis is triggered by CBP hyperphosphorylation, a modification mediated by CDK activation and / or PP6 inactivation.

In separate studies, we determined that besides being necessary for HK-mediated neuronal survival, NF-κB is also required for the survival-promoting effects of IGF-1, cyclic AMP, and lithium as judged by the ability of SN50 to inhibit the actions of these survival factors, and the ability of these factors to inhibit the reduction of DNA-binding activity of NF-κB resulting from LK-treatment.

2. IκB-β phosphorylation and neuronal survival.

We found that IκB-β is phosphorylated in healthy neurons. LK treatment causes a reduction in the extent of IκB-β phosphorylation. To map the phosphorylation site and identify the kinase involved in acting on it we generated wild-type, truncated, and point-mutant forms of IκB-β expressed as GST-fusion proteins and used these in *in vitro* kinase assays. We found that HK-mediated phosphorylation of IκB-β is not mediated by IκB kinase (IKK), but by the nonreceptor tyrosine kinase Abl2 (Arg). Phosphorylation by Abl2 occurs at a novel site, Tyr161. Consistent with Abl-mediated phosphorylation, we found that the treatment of neurons with highly selective Abl inhibitors, PD173955 and Gleevec blocks HK-induced phosphorylation of IκB-β at Tyr161. Treatment with these inhibitors also induces neuronal apoptosis in HK medium suggesting that IκB-β phosphorylation is necessary for HK-mediated survival. In contrast to phosphorylation by IKK, which is known to inhibit NF-κB activity, the phosphorylation of IκB-β by Abl2 enhances its association with NF-κB and stimulates NF-κB DNA-binding activity. In addition to the known roles for Abl2 in neuronal

morphogenesis and axon guidance during nervous system development, our findings implicate this kinase in the maintenance of neuronal survival. Thus, in addition to CBP hyperphosphorylation, the activity of NF-κB is lowered during neuronal apoptosis by a reduction in the phosphorylation of IκB-β. Preliminary results indicate that CBP, p65 and IκB-β exist as a complex in HK medium.

3. Akt as a potential convergent point in survival-promotion signaling

In our request for supplementary funding submitted in 2000, we had hypothesized that Akt was required for the survival-promoting effect of HK, IGF-1, cyclic AMP, and lithium on CGNs. We proposed to examine if the four factors stimulated Akt phosphorylation and activity, respectively. Consistent with it being a convergent point in signaling by different survival-promoting factors, we found that Akt is phosphorylated and activated by all four survival factors. The pattern of Akt phosphorylation induced by the four survival factors, however, showed differences. While IGF-1 induced phosphorylation of Akt at both Ser473 and Thr308, HK and cyclic AMP stimulate phosphorylation at Thr308 only. Lithium increased phosphorylation at Ser473 but not at Thr308. The different phosphorylation patterns point to a previously unappreciated complexity in the regulation of Akt activity in neurons. To more directly examine the role of Akt in neuronal survival by the different factors we inhibited it using pharmacological inhibitors and by overexpression of an adenoviral-delivered dominant-negative Akt construct. These experiments demonstrated that Akt is required for the survival mediated by IGF-1 and lithium. Survival by HK or cyclic AMP, however, is Akt-independent.

Our work showed that Akt is not a molecule that is indispensable to neuronal survival. Since IGF-1 and lithium, but not HK or cAMP, stimulated phosphorylation of Akt at Ser473, phosphorylation of this residue might be of particular significance to Akt-mediated neuronal survival. Despite efforts by numerous labs, the kinase responsible for Ser473 phosphorylation has not been identified. In other experiments, we found that serum-glucocorticoid regulated kinase (SGK, a kinase that is structurally related to Akt, is also activated by the four survival factors. It is possible that SGK is required for the survival-promoting effect of HK and cyclic AMP in CGNs. SGK may also be of significant importance to the survival of other neuronal types.

4. The relationship between Akt and NF-κB

Although initially believed to operate as components of distinct signaling pathways, several studies have demonstrated that the NF-κB and Akt signaling pathways can converge. Results from these studies placed Akt upstream of NF-κB activation. Unexpectedly, we found that the treatment of neurons with pharmacological inhibitors of NF-κB, such as SN-50 and TPCK, blocked the ability of IGF-1 to phosphorylate Akt indicating that Akt might act downstream of NF-κB in the survival-promoting pathway.

To test the hypothesis that Akt was a downstream target of NF-κB, we initially used non-neuronal cells. It has been well-established that the treatment of NIH 3T3 cells with the NF-κB activators, TNFα and lipopolysaccharide (LPS), results in the stimulation of Akt phosphorylation. In carefully performed time-course experiments, we found that the stimulation of Akt is detected only after IκB-άdegradation (an event necessary for NF-κB activation) is induced by these agents. The nuclear translocation of p65 and increased DNA-binding activity of NF-κB also precede Akt phosphorylation. Treatment of these cultures with the NF-κB inhibitors, SN50 and TPCK, blocked TNF-induced Akt activation. On the other hand, TNF-mediated NF-κB activation was not reduced by the phosphoinositide-3

kinase (PI-3K) inhibitors wortmannin and LY294002 although these inhibitors completely blocked the activation of Akt. These results were consistent with our hypothesis that NF-κB is required for TNF-mediated Akt activation and that it lies upstream of Akt. Further strengthening our conclusion was the finding that overexpression of p65 led to Akt phosphorylation in the absence of extracellular stimulatory factors, while overexpression of IκB-α reduced Akt phosphorylation below basal levels. Our results using NIH3T3 cells were confirmed in primary cultures of vascular endothelial cells.

We proceeded to examine whether PDK1 (a serine-threonine kinase that phosphorylates and activates Akt) was an intermediary between NF-κB and Akt. We found that elevated p65 expression led to an activation of PDK1. Treatment with antisense PDK1 oligonucleotides reduced PDK1 expression and inhibited the stimulation of Akt phosphorylation by p65. We also found that p65-mediated activation of PDK1 was mediated by PI-3 kinase. Thus, elevated p65 expression activated PI-3 kinase whereas pharmacological inhibition of PI-3 kinase blocked both PDK1 and Akt phosphorylation by p65.

Interestingly, in addition to stimulating the phosphorylation of Akt, the overexpression of p65 caused an increase in the expression of Akt mRNA and protein. We found that both effects (increased phosphorylation and expression) were blocked by transcriptional and translational inhibitors, suggesting the need for new gene expression. In contrast to p65-mediated Akt phosphorylation however, neither the inhibition of PI-3 kinase, nor of PDK1, affected the ability of p65 to stimulate Akt expression. This suggests that distinct mechanisms underlie the two stimulatory effects of p65 on Akt. We cloned the promoter of the Akt1 gene, linked it to a luciferase reporter, and used the construct in transcriptional assays. We found that the transcriptional activity of the Akt1 promoter is robustly stimulated by co-transfection with CMV-p65 but not by a control LacZ expressing vector, demonstrating that the stimulation of Akt expression by p65 is mediated at the transcriptional level.

We examined whether overexpression of Akt could rescue neurons from death caused by pharmacological inhibition of NF-κB. The rational here was that if NF-κB promoted neuronal survival by activating Akt, overexpression of Akt would maintain neuronal survival even if NF-κB was inhibited. We found that although survival in IGF-1 is blocked by the inhibition of NF-κB by SN-50 treatment, the neurons are fully rescued if infected with Ad-Akt. This result confirms that Akt lies downstream of NF-κB in the survival-promoting signaling pathway utilized by IGF-1 in neurons.

5. Analysis of GW5074 as a neuroprotective compound

Our request for supplementary funding in 2002 was to study a remarkable chemical compound called GW5074 {5-Iodo-3-[(3,5-dibromo-4-hydroxyphenyl) methylene]-2-indolinone}. GW5074 is a commercially available chemical inhibitor of c-Raf. We had observed (and then confirmed) that treatment with GW5074 could prevent LK-induced death of CGNs. GW5074 had no direct effect on the activities of several apoptosis-associated kinases when assayed *in vitro*. Surprisingly however, and in contrast to its effect *in vitro*, treatment of neurons with GW5074 caused c-Raf activation (when measured *in vitro* in the absence of the drug) and stimulated the Raf-MEK-ERK pathway. Treatment of neurons with GW5074 also led to an increase in the activity of B-Raf, which was not efficiently inhibited by GW5074 *in vitro* at concentrations at which the drug exerts its neuroprotective effect. PD98059 and U0126, two distinct inhibitors of MEK, blocked the activation of ERK by GW5074 but had no effect on its ability to prevent cell death. Overexpression of a dominant-

negative form of Akt did not reduce the efficacy of GW5074, demonstrating an Akt-independent mechanism of action. Neuroprotection was inhibited by SN-50, a specific inhibitor of NF-κB and by the Ras inhibitor S-trans, trans-farnesylthiosalicylic acid (FTS) implicating NF-κB and Ras in the neuroprotective signaling pathway activated by GW5074. In addition to preventing LK-induced apoptosis, treatment with GW5074 protected against the neurotoxic effects of MPP+ and methylmercury in CGNs , and glutathione depletion-induced oxidative stress in cortical neurons. Furthermore, GW5074 prevented neurogeneration and improved behavioral outcome in an animal model of Huntington's disease. Given its neuroprotective effect on distinct types of cultured neurons, in response to different neurotoxic stimuli, and in an animal model of neurodegeneration, GW5074 could have therapeutic value against neurodegenerative pathologies in humans.

6. 3' substituted indolone as a scaffold for the development of neuroprotective drugs.

In more recent studies we focused on another chemical compound called GW8510 4-{[(7-Oxo-6,7-dihydro-8H-[1,3]thiazolo[5,4-e]indol-8 ylidene)methyl] amino}-N-(2-pyridinyl)-benzenesulfonamide} was developed recently as an inhibitor of cyclin-dependent kinase 2 (CDK2). We found that GW8510 also inhibited death CGNS caused by LK treatment. Surprisingly, we found that although GW8510 inhibited CDK2 and other CDKs when tested in in vitro biochemical assays, when used on cultured neurons it only inhibited CDK5, a cytoplasmic CDK that is not associated with cell-cycle progression. Treatment of cultured HEK393T cells with GW8510 did not inhibit cell-cycle progression, consistent with its inability to inhibit mitotic CDKs in intact cells. We determined that neuroprotection by GW8510 was independent of Akt and MEK-ERK signaling. Furthermore, GW8510 did not block the LK-induced activation of GSK3β, and while inhibiting c-jun phosphorylation, it did not inhibit the increase in c-jun expression observed in apoptotic neurons.

Like GW5074, GW8510 is a 3' substituted indolone raising the possibility that the 3' substituted core that these two compounds share might be important for their ability to neuroprotect. To test this idea, we examined the effectiveness of other 3' substituted indolone compounds to protect against neuronal apoptosis. We found that like GW8510, the VEGF Receptor 2 kinase inhibitors [3-(1H-pyrrol-2-ylmethylene)-1,3-dihydroindol-2-one], {(Z)-3-[2,4-Dimethyl-3-(ethoxycarbonyl)pyrrol-5 yl)methylidenyl]indol-2-one}, and [(Z)-5-Bromo-3-(4,5,6,6-tetrahydro-1H-indol-2-ylmethylene)-1,3-dihydroindol-2-one], the Src family kinase inhibitor SU6656, and a commercially available inactive structural analog of an RNAdependent protein kinase inhibitor 5-Chloro-3-(3,5-dichloro-4-hydroxybenzylidene)-1,3-dihydro-indol-2-one, are all neuroprotective when tested on LK-treated neurons.

Our findings identify the 3' substituted indolone as a core structure for the designing of neuroprotective drugs that may be used to treat neurodegenerative diseases in humans.

KEY RESEARCH ACCOMPLISHMENTS:

- NF-κB is a molecule that is pivotal for neuronal survival. Moreover, the activity of NF-κB in neurons is regulated by its interaction with CBP. Phosphorylation of CBP leads to reduced association between NF-κB and CBP leading to neuronal death.
- Akt is activated by different survival-promoting factors. While it is necessary for survival-promotion by some factors (such as IGF-1 and lithium), it is not required for neuronal survival by other agents (such as HK or cAMP).
- In neurons treated with IGF-1, Akt serves as a downstream target of NF-κB.

- GSK3β is a protein that is necessary for neuronal death, while PAK-1 is a molecule that is likely to promote neuronal survival.
- GW5074 is a small-molecule neuroprotective compound that can prevent neurodegeneration is tissue culture and in vivo models of neurodegeneration.
- 3' substituted indolones represent a scaffold for the rational design of therapeutic compounds to treat neurodegenerative conditions.

REPORTABLE OUTCOMES:
Peer-reviewed publications

1) Koulich, E, Thuyen N, Johnson K, Giardina CA, D'Mello, SR. (2001) NF-κB is involved in the survival of cerebellar granule neurons: Association of IκB-β phosphorylation with cell survival. *J. Neurochem*. 76, 1188-1198.

2) Kumari S, Liu X, Nguyen T, D'Mello SR. (2001) Distinct patterns of Akt phosphorylation by survival factors in cerebellar granule neurons. *Mol. Brain Res.* 97, 157 - 162.

3) Meng F, Liu L, Chin PC, D'Mello SR. (2002) Akt is a downstream target of NF-κB. *J. Biol. Chem*. 277, 29674-29680.

4) Yalcin A, Koulich E, Mohamed S, Liu L, D'Mello SR. (2003) Apoptosis in cerebellar granule neurons is associated with CBP hyperphosphorylation and reduced interaction between CBP and NF-κB *J. Neurochem*. 84, 397 - 408.

5) Meng F and D'Mello SR. (2003) NF-κB stimulates Akt phosphorylation and gene expression by distinct signaling mechanisms *Biochem. Biophys. Acta,* 1630, 35 - 40.

6) Chin PC and D'Mello SR. (2004) Survival of cultured cerebellar granule neurons can be maintained by Akt-dependent and Akt-independent signaling pathways. *Mol. Brain Res.* 127:140-145.

7) Chin PC, Liu L, Morrison B, Bottiglieri T, Ratan RR, D'Mello SR. (2004) The c-Raf inhibitor GW5074 provides neuroprotection in vitro and in an animal model of neurodegeneration through a MEK-ERK and Akt-independent mechanism. *J. Neurochem*. 90, 595-608.

8) Johnson K and D'Mello SR. (2005) PAK-1 is necessary depolarization-mediated neuronal survival. *J. Neuroscience Res.*79, 809-815.

9) Johnson K, Liu L Majdazeh N, Chavez C, Chin PC, Morrison B, Wang L, Park J, Chugh P, ChenH-M, D'Mello SR (2005) Inhibition of neuronal apoptosis by the cyclin dependent kinase inhibitor GW8510: Identification of 3' substituted indolones as a scaffold for the development of neuroprotective drugs. *J. Neurochem.* 93, 538 - 548.

10) Chin PC, Majdazeh N, D'Mello SR (2005) Neuroprotection by distinct survival factors involves the inhibition of Gsk3β. *Mol. Brain Res.* 137, 193-201.

11) Liu L and D'Mello SR. (2006) Phosphorylation of IκB-β is necessary for neuronal survival. *J. Biol. Chem.*281, 1506 – 1515.

12) Morrison BE, Majdzadeh N, Zhang X, Lyles L, Bassel-Duby R, Olson EN, D'Mello SR Neuroprotection by histone deacetylase-related protein. *Mol. Cell Biol. (in press).*

Reviews published:

1) Chin PC, D'Mello SR. (2005) Brain chemotherapy from the bench to the clinic: targeting neuronal survival with small molecule inhibitors of apoptosis. *Front Biosci.* 10, 552-568.

2) D'Mello SR, Chin PC. (2005) Treating neurodegenerative conditions through the understanding of neuronal apoptosis. *Curr Drug Targets CNS Neurol Disord.* 4, 3-23.

Training:

Funds from the grant have been used to support two postdoctoral fellows over the 6 year period. A research technician was also supported. Additionally, two graduate students were trained using funds from this grant.

CONCLUSIONS

The research performed with DAMD funding has shed new insight into the molecular mechanisms underlying the regulation of neuronal survival. Moreover, our studies have led to the identification of neuroprotective compounds that might have value in the treatment of neurodegenerative conditions in humans.

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Short communication

Survival of cultured cerebellar granule neurons can be maintained by Akt-dependent and Akt-independent signaling pathways

Paul C. Chin, Santosh R. D'Mello*

Department of Molecular and Cell Biology, University of Texas at Dallas, 2601 N. Floyd Road, Richardson, TX 75083, USA

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Abstract

Cerebellar granule neurons can be maintained in culture by four factors: depolarizing levels of potassium (HK, 25 mM), cyclic AMP elevating agents and analogs (cyclic adenosine monophosphate, cAMP), insulin-like growth factor-1 (IGF-1), or lithium. We investigated the possibility that the signal transduction pathways utilized by these four survival factors might converge at a common molecular target and that the serine/threonine kinase Akt might be the convergent molecule. Previous research demonstrated that the four factors could phosphorylate and activate Akt; thus, using pharmacological inhibition of Akt and overexpression of an adenoviral delivered dominant negative Akt construct, we analyzed the role of Akt in the survival mediated by each factor. We found that although Akt is required for the survival mediated by IGF-1 and lithium, it is dispensable for the survival mediated by high potassium and cAMP.

Theme: Development and regeneration
Topic: Neuronal death

Keywords: Akt; IGF-1; HK; Cyclic AMP; Lithium; Cerebellar granule neuron; Apoptosis

In the development of the mammalian nervous system, superfluous neurons are eliminated by apoptosis. While being critical for normal neurodevelopment, deregulated apoptosis contributes to the excessive neuronal loss observed in a variety of neurodegenerative diseases, and following stroke or traumatic head injury (for review, Refs. [7,36,54]). Much attention has therefore been focused on understanding the intracellular signaling pathways that promote or inhibit neuronal apoptosis. A commonly used cell culture paradigm that has been used to understand the molecular mechanisms underlying apoptosis is cultured cerebellar granule neurons. These cultures remain viable in the presence of depolarizing levels of extracellular potassium (K+), a stimulus that mimics neuronal activity, which is known to be important for the survival of several neuronal types in vivo [17]. Switching of granule neuron cultures from a high K^+ (HK) medium to one with low K^+ (LK, 5 mM) induces apoptosis [8,38,52]. LK-induced apoptosis can be inhibited, and neuronal survival maintained by insulin-like growth factor-1 (IGF-1), a growth factor with well-established neurotrophic activity for many neuronal types including cerebellar granule neurons in vivo [32,53]. Besides trophic factor and depolarizing stimuli, the survival of granule neurons can be mediated by cyclic AMP and lithium [8–10]. Like cerebellar granule neurons, the survival of other neuronal populations can also be supported in vitro by HK, cyclic AMP, lithium, and specific growth factors [13,18,25,43,44,51].

HK-treatment causes membrane depolarization leading to an influx of calcium through voltage-gated channels initiating HK-mediated survival [16,28]. In contrast, growth factors such as IGF-1 and nerve growth factor (NGF) activate receptor tyrosine kinases and promote neuronal survival by a phosphatidylinositide-3 kinase (PI-3K)-dependent pathway [5,10–12,20,37]. In cerebellar granule neurons, survival by HK is PI-3K-independent [10,12], although this conclusion has been contradicted by some

Abbreviations: cAMP, cyclic adenosine monophosphate; CREB, cAMP response element binding protein; GSK-3, glycogen synthase kinase-3; MOI, multiplicity of infection; NF-κB, nuclear factor-κB; PI-3K, phosphatidylinositide-3 kinase

* Corresponding author. Tel.: +1-972-883-2520; fax: +1-972-883-2409.
E-mail address: dmello@utdallas.edu (S.R. D'Mello).

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studies [37,46]. Neuronal survival by cyclic adenosine monophosphate (cAMP), however, does not require either elevated intracellular calcium or PI-3K activation [5,39,41]. Furthermore, lithium-mediated survival occurs via an incompletely understood mechanism, but survival most likely involves regulation of inositol monophosphatase, inositol polyphosphatase, Akt, and glycogen synthase kinase-3 (GSK-3) [1,2,27,35,49].

While clearly activating distinct upstream molecules, it is likely that the signaling pathways activated by these different survival-promoting molecules converge on a common downstream target. Identifying such a target would increase our basic understanding of neuronal survival and would aid in developing preventative and therapeutic strategies against neurodegenerative diseases. One potential common downstream target is the 60 kDa serine/threonine kinase Akt or protein kinase B (PKB). Akt contributes to protecting cells from apoptosis against a variety of apoptotic insults (for review, Refs. [24,45]). Previous work by Kumari et al. [31] has shown that HK, IGF-1, cAMP and lithium activate Akt in cultured cerebellar granule neurons, supporting the idea of Akt as the convergent molecule. In this study, we have used cultured cerebellar granule neurons to examine the necessity of Akt in the survival effects of HK, IGF-1, cAMP, and lithium. We report that IGF-1 and lithium-mediated neuronal survival is dependent on Akt. In contrast, survival by HK and cyclic AMP is mediated by an Akt-independent mechanism.

Unless otherwise specified, all materials were purchased from Sigma (St. Louis, MO). All antibodies were obtained from Cell Signaling Technologies (Beverly, MA). IGF-1 was purchased from Roche Biochemicals (Indianapolis, IN). ML-9 was purchased from Calbiochem (La Jolla, CA). The recombinant adenovirus expressing green fluorescent protein (Ad-GFP) was a gift from Kim A. Heidenreich (University of Colorado Health Sciences Center, Denver, CO), and the adenoviral expression vector encoding a hemagglutinin-tagged (HA) dominant-negative Akt (Ad-dnAkt) was a gift from Wataru Ogawa (Kobe University, Hyogo, Japan).

Granule neuron cultures were obtained from dissociated cerebella of 7–8-day-old rats as described previously [8]. Cells were plated in Basal Eagle's medium with Earles salts (BME) supplemented with 10% fetal bovine serum (FBS), 25 mM KCl, 2 mM glutamine (Gibco-BRL), and 100 μg/ml gentamycin on dishes coated with poly-L-lysine in 24-well dishes at a density 1.0×10^6 cells/well or 1.0×10^7 cells/60 mm dish. Cytosine arabinofuranoside (10 μM) was added to the culture medium 18–22 h after plating to prevent replication of non-neuronal cells. Unless indicated otherwise, cultures were maintained for 6–7 days prior to experimental treatments. For this, the cells were rinsed once and then maintained in low K^+ medium (serum-free BME medium, 5 mM KCl) with or without the agents, or in high K^+ medium (serum-free BME medium, supplemented with 20 mM KCl). The final concentrations of various factors and agents were as follows: 25 mM KCl in HK medium, 50 ng/ml IGF, 10 μM forskolin, and 10 mM lithium chloride.

Neuronal survival was quantified by the MTT assay as previously described [29]. Briefly, the tetrazolium salt MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide] was added to the cultures at a final concentration of 1 mg/ml, and incubation of the culture was continued in the CO_2 incubator for a further 30 min at 37 °C. The assay was stopped by adding lysis buffer [20% SDS in 50% *N*,*N*-dimethyl formamide, pH 4.7]. The absorbance was measured spectrophotometrically at 570 nm after an overnight incubation at room temperature. The absorbance of a well without cells was used as background and subtracted. Results obtained using the MTT assays were confirmed by quantifying apoptotic cells following 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) staining. For this, the proportion of apoptotic cells (condensed and fragmented nuclei) was calculated as a percentage of the total cells counted from 5 microscopic fields ($\times 400$). Data are presented as mean ± standard deviation (S.D.). Statistical analysis was performed using ANOVA and Student–Neuman–Keuls' test.

To prepare whole-cell lysates for Western blots, the culture medium was discarded, the neurons washed twice with ice-cold phosphate-buffered saline (PBS), and lysed in lysis buffer (1% Triton, 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 1X protease inhibitor mixture). Protein concentrations were measured using a Bradford protein assay kit (Bio-Rad), and equivalent amounts of protein were mixed with 6× SDS-PAGE sample buffer. Following heating at 95 °C for 5 min, proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene difluoride membrane (PVDF; Bio Rad). After staining with Ponceau S to verify uniformity of protein loads/transfer, the membranes were analyzed for immunoreactivity. Incubation with primary antibodies was performed overnight at 4 °C and with secondary antibodies for 1 h at room temperature. Immunoreactivity was developed by enhanced chemiluminescence (ECL; Amersham) and visualized by autoradiography.

The hemagglutinin-tagged (HA) dominant-negative Akt (Ad-dnAkt) consists of two mutations where the phosphorylation sites at threonine 308 (T308) and serine 473 (S473) are mutated to yield a phosphorylation-deficient inactive protein. Ad-dnAkt and Ad-GFP are propagated in HEK293T cells and purified by cesium chloride density gradient centrifugation. After quantification of titer, virus at a multiplicity of infection (MOI) of 50 are used to infect granule neuron cultures 5 days after plating by direct addition to the medium. Treatments are performed 24 h after addition of virus. Infected neurons were detected by GFP fluorescence or by positive staining for HA by immunocytochemistry procedures as described previously [29]. The proportion of

apoptotic cells (condensed or fragmented nuclei) as a percentage of total infected neurons was quantified following DAPI-staining.

When cultures of cerebellar granule neurons are switched from media containing depolarizing levels of potassium, HK, to media containing physiological concentrations of K+ (5 mM, referred to as low potassium, LK), they die by apoptosis [8,38,52]. At 8 h following a switch to LK, neurons begin to exhibit characteristic apoptotic morphological changes including cellular shrinkage, chromatin condensation and nucleosomal fragmentation. At 24 h following LK-treatment, 50% of the neurons will have undergone apoptosis. Our lab and others have found that cyclic AMP elevating agents and analogs, IGF-1, and lithium can maintain the survival of cultures although not as effectively as HK [8–10,16,52]. In addition, we recently showed that all four survival factors activate Akt. In an effort to determine the necessity of Akt in the survival mediated by each factor we first used ML-9, which has been used as pharmacological inhibitor of Akt in adipocytes, myoblasts, endothelial, and hepatocellular carcinomas cells [6,19,47,48].

In cerebellar granule neurons, as shown in Fig. 1, increasing concentrations of ML-9 lead to decreases in the levels of Akt phosphorylation at S473 in response to IGF-1 stimulation. These decreases in Akt phosphorylation coincide with decreases in the levels of phosphorylated GSK-3β. This suggests that the activity of Akt is reduced by pharmacological inhibition using ML-9, given that GSK-3β is downstream of Akt in the signaling directed by IGF-1 [3,4,21,23,42].

After demonstrating that ML-9 could reduce the levels of phosphorylated Akt and GSK-3β, thus affecting signaling through Akt, we examined the survival of granule neurons with each of the four survival factors in the presence of ML-9. Fig. 2 depicts the effects of Akt inhibition with ML-9 in

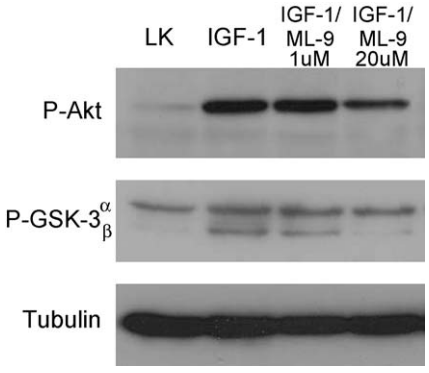

Fig. 1. ML-9 inhibits Akt. Seven-day-old cerebellar granule neuron cultures were switched to LK media for 2 h to cause downregulation of basal Akt activity. A 50 ng/ml IGF-1 was then added for 15 min to stimulate Akt activity. Two cultures were treated with ML-9 30 min prior to stimulation with IGF-1. Akt and GSK-3 phosphorylation were measured by Western blot using phosphorylation-specific antibodies. The same blot was then probed with a tubulin antibody. Similar results were obtained in three separate experiments.

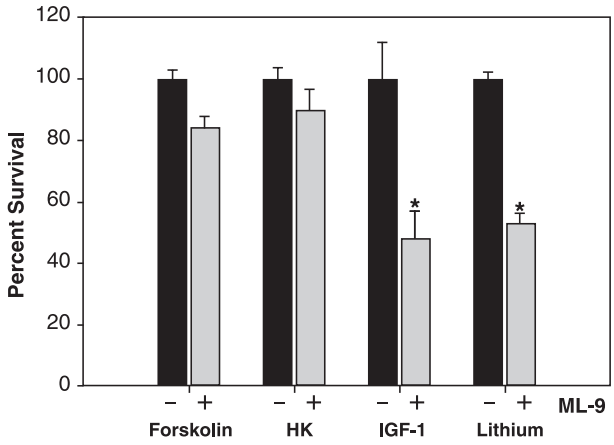

Fig. 2. Pharmacological inhibition of Akt reduces survival mediated by IGF-1 and lithium. Cerebellar granule neurons were treated with 10 μM forskolin, 25 mM HK, 50 ng/ml IGF-1, or 10 mM lithium in the absence or presence of ML-9 at 20 μM. Cell viability was measured by the MTT assay 24 h later and is expressed as a percentage of the HK control. Data represent the means ± S.D. from three separate experiments, each performed in duplicate. * indicates significance at $p < 0.001$. Similar results were obtained using DAPI-staining to quantify apoptotic cells (condensed or fragmented nuclei).

the survival mediated by forskolin, HK, IGF-1, and lithium. For these experiments, cultures were switched to media containing one of the four survival factors in the presence or absence of ML-9, and the viability was measured 24 h later. Both IGF-1 and lithium-mediated survival were dramatically decreased following incubation with ML-9 while forskolin, which increases intracellular cyclic AMP levels, and HK-mediated survival were only slightly affected.

A drawback with using ML-9 to inhibit Akt is that ML-9 has been shown to inhibit a variety of other kinases including PKA, PKC, and myosin light chain kinase (MLCK), and the results obtained could reflect inhibition other than Akt. Moreover, recent work by some groups has lead to the development of three potent and selective phosphatidylinositol analogue Akt inhibitors [22,30]. However, in our hands, two of these inhibitors had little to no effect on Akt activation or survival mediated by IGF-1 (data not shown). Therefore, to more accurately predict the necessity of Akt in the survival directed by each factor, we infected granule neuron cultures with an adenoviral vector expressing an Akt functional mutant (Ad-dnAkt).

Twenty-four hours after infecting granule neurons cultures with Ad-dnAkt or an Ad-GFP control vector at an MOI of 50, the culture medium was replaced with forskolin, HK, IGF-1, or lithium for 24 h. As seen in Fig. 3, blockade of Akt signaling causes apoptosis in nearly 60% of infected neurons in IGF-1 signaling, similar to previous reports [12]. In addition, blockade of Akt signaling resulted in the death of 70% of the infected neurons in the survival directed by lithium. On the other hand, functional inhibition of Akt in HK or forskolin had no effect on survival, suggesting that although Akt may contribute to survival in HK and forskolin, it is not required to mediate survival.

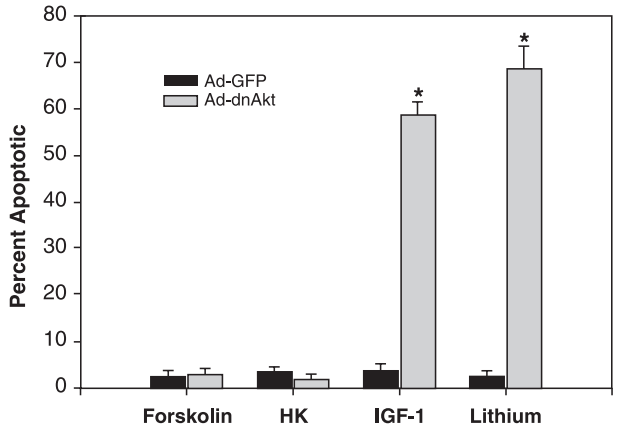

Fig. 3. Akt is necessary for survival mediated by IGF-1 and lithium, but dispensable for forskolin and HK. Five-day-old neuronal cultures were infected with adenoviral vectors expressing either GFP or hemagglutinin (HA)-tagged dominant-negative Akt. The next day the cultures were switched to LK medium containing 10 μM forskolin, 25 mM HK, 50 ng/ml IGF-1, or 10 mM lithium for 24 h. Infected neurons were detected by GFP fluorescence or positive staining for HA by immunocytochemistry, and following DAPI staining, the proportion of apoptotic cells (condensed or fragmented nuclei) as a percentage of total infected neurons was quantified. Data represent the means ± S.D. from three separate experiments, each performed in duplicate. * indicates significance at $p < 0.001$.

Akt is a potent survival-promoting molecule that has been shown to be necessary for survival of a variety of neurons and non-neuronal cells (for review, Refs. [24,45]). Akt is activated by phosphorylation of two important sites, T308 and S473. Mutational analysis has demonstrated that phosphorylation of T308 is sufficient to activate Akt, but phosphorylation of both residues is required for maximal activation [24,45]. In cerebellar granule neurons, Akt is phosphorylated by HK, IGF-1, cyclic AMP, and lithium, though by distinct patterns; while HK and cyclic AMP stimulate phosphorylation at T308 only, treatment with lithium phosphorylates S473 but not T308 [31]. IGF-1 on the other hand, stimulates phosphorylation at both residues. Despite the differences in the pattern of phosphorylation, all four survival-promoting agents activate Akt activity [31]. We report that the survival-promoting effects of IGF-1 and lithium are dependent on Akt. Treatment with a pharmacological agent that causes reduction of Akt activity and direct inhibition of Akt by overexpression of a dominant-negative construct dramatically reduce the effectiveness of IGF-1 and lithium to maintain survival. In contrast, HK and cAMP are not affected by Akt inhibition. Li et al. [33] have also reported that cAMP-mediated neuronal survival occurred by an Akt-independent manner. Taken together, these observations raise the possibility that phosphorylation of S473 is of particular significance to the ability of Akt in sustaining neuronal survival. The precise mechanism by which this may be mediated is unclear. It is conceivable that phosphorylation at S473 could permit Akt to interact with specific molecules. Interaction between Akt and the scaffold protein JNK-interacting protein (JIP) leading to inhibition of Jun-N-terminal kinase (JNK) and thus c-Jun activity has previously been reported [26].

Assuming that the survival pathways activated by the different survival factors do converge, our results indicate that although a potent survival-promoting molecule, Akt is not a convergent point. Another possible candidate is GSK-3. GSK-3 is activated during apoptosis in many paradigms and inhibition of GSK-3 has been shown to promote cell survival (for review, Ref. [15]). Activation of GSK-3 has been shown to be a downstream target of Akt and is thus inactivated by neurotrophic factors such as IGF-1 and NGF [3,4,21,23,42]. In addition, GSK-3 is inhibited by PKA in cerebellar granule neurons and other cell types [14,33]. Finally, the ability of lithium to inhibit GSK-3 is well established [2,9,40]. Nuclear factor-κB (NF-κB) represents another potential convergent point [29,50]. HK, cAMP, and IGF-1 stimulate the binding activity of NF-κB and the effectiveness of these factors to promote survival of granule neurons is blocked by pharmacological inhibition of NF-κB [29]. Yet another candidate is the cAMP response element binding protein (CREB). Both HK and growth factors have been shown to phosphorylate and activate CREB (for review, Ref. [34]).

In summary, using a combination of pharmacological and molecular biological approaches we demonstrate that neuronal survival can be mediated by Akt-dependent or Akt-independent mechanisms. The signaling pathway by which Akt sustains the survival of neurons and non-neuronal cells has been under intense investigation. Identifying the molecular components of the Akt-independent pathway would further our understanding of how neuronal survival is regulated and would provide additional avenues for the development of therapeutic strategies for neurodegenerative diseases.

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The c-Raf inhibitor GW5074 provides neuroprotection *in vitro* and in an animal model of neurodegeneration through a MEK-ERK and Akt-independent mechanism

Paul C. Chin,* Li Liu,* Bradley E. Morrison,* Ambreena Siddiq,† Rajiv R. Ratan,†,‡ Teodoro Bottiglieri§ and Santosh R. D'Mello*

**Department of Molecular and Cell Biology, University of Texas at Dallas, Richardson, Texas, USA*

†*Department of Neurology, Harvard Medical School and The Beth Israel Deaconess Medical Center, Boston, Massachusetts, USA*

‡*Department of Neurology and Neuroscience, Weil Medical College of Cornell University, New York, USA*

§*Baylor Institute of Metabolic Disease, Dallas, Texas, USA*

Abstract

Cerebellar granule neurons undergo apoptosis when switched from a medium containing high potassium (HK) to one that has low potassium (LK). LK-induced cell death is blocked by GW5074 {5-Iodo-3-[(3,5-dibromo-4-hydroxyphenyl) methylene]-2-indolinone}, a synthetic drug that inhibits c-Raf activity *in vitro*. GW5074 has no direct effect on the activities of several apoptosis-associated kinases when assayed *in vitro*. In contrast to its effect *in vitro*, treatment of neurons with GW5074 causes c-Raf activation (when measured *in vitro* in the absence of the drug) and stimulates the Raf-MEK-ERK pathway. Treatment of neurons with GW5074 also leads to an increase in the activity of B-Raf, which is not inhibited by GW5074 *in vitro* at concentrations at which the drug exerts its neuroprotective effect. PD98059 and U0126, two distinct inhibitors of MEK, block the activation of ERK by GW5074 but have no effect on its ability to prevent cell death. Overexpression of a dominant-negative form of Akt does not reduce the efficacy of GW5074, demonstrating an Akt-independent mechanism of action. Neuroprotection is inhibited by SN-50, a specific inhibitor of nuclear factor-kappa B (NF-κB) and by the Ras inhibitor S-*trans*, *trans*-farnesylthiosalicylic acid (FTS) implicating NF-κB and Ras in the neuroprotective signaling pathway activated by GW5074. In addition to preventing LK-induced apoptosis, treatment with GW5074 protects against the neurotoxic effects of MPP+ and methylmercury in cerebellar granule neurons, and glutathione depletion-induced oxidative stress in cortical neurons. Furthermore, GW5074 prevents neurodegeneration and improves behavioral outcome in an animal model of Huntington's disease. Given its neuroprotective effect on distinct types of cultured neurons, in response to different neurotoxic stimuli, and in an animal model of neurodegeneration, GW5074 could have therapeutic value against neurodegenerative pathologies in humans.

Keywords: B-Raf, cerebellar granule neurons, Huntington's disease, neurodegeneration, neuroprotection.

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Aberrant apoptosis is a common feature in a variety of neurodegenerative diseases, in neuropathological conditions such as stroke and following traumatic brain injury. Much of our knowledge of how neuronal apoptosis is regulated has come from *in vitro* paradigms using primary cultures of neurons, and several molecules involved in promoting neuronal apoptosis have been identified (reviewed in Deshmukh and Johnson 1997; D'Mello 1998; Chang *et al*. 2002; Mattson 2000). Among these is the transcription factor c-jun, which is phosphorylated and activated during the apoptotic process (Estus *et al*. 1994; Ham *et al*. 1995; Watson *et al*. 1998). Phosphorylation of c-jun is mediated by jun

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Address correspondence and reprint requests to Santosh R. D'Mello, Department of Molecular and Cell Biology, University of Texas at Dallas, 2601 N. Floyd Road, Richardson, TX 75083, USA.
E-mail: dmello@utdallas.edu

Abbreviations used: Cdk, cyclin-dependent kinases; DAPI, 4′,6′-diamidino-2-phenylindole hydrochloride; DTT, dithiothreitol; ERK, extracellular signal-regulated protein kinase; FTS, S-*trans*, *trans*-farnesylthiosalicylic acid; HCA, homocysteate; JNK, c-Jun N-terminal kinase; MEK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappa B; 3-NP, 3-nitropropionic acid; PI-3 kinase, phosphatidylinositol 3-kinase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

N-terminal kinase (JNK; Eilers *et al.* 1998), which can be encoded by three genes- JNK1, JNK2, and JNK3 (reviewed in Barr and Bogoyevitch 2001; Weston and Davis 2002). Although the JNKs can be activated *in vitro* by MKK4 or MKK7, only MKK7 appears to be involved in stimulating c-jun phosphorylation during neuronal apoptosis (Eilers *et al*. 1998; Trotter *et al*. 2002). Members of the mixed lineage kinase (MLK) family lie upstream of MKK4 and MKK7 (Xu *et al*. 2001; Harris *et al*. 2002). Several lines of evidence also implicate an abortive re-entry into the cell cycle caused by activation of certain cyclin-dependent kinases (Cdks) as a critical feature of neuronal apoptosis (reviewed in Copani *et al*. 2001; Liu and Greene 2001; O'Hare *et al*. 2002).

In the presence of survival-promoting stimuli such as neuronal activity or neurotrophic growth factors, the activation of pro-apoptotic molecules is blocked. One signaling pathway involved in the promotion of growth factor-mediated neuronal survival is the phosphatidylinositol 3-kinase (PI-3K)-Akt pathway (Datta *et al*. 1997; Dudek *et al*. 1997; Crowder and Freeman 1998). Once activated, Akt phosphorylates a number of pro-apoptotic molecules including the Bcl-2 protein BAD, the Forkhead transcription factor, glycogen synthase kinase-3 (GSK-3) and caspase-9 (for a review, see Brunet *et al*. 2001) leading to their inactivation. Another signaling pathway that has been implicated in the promotion of neuronal survival, at least in some systems, is the Raf-MEK-ERK pathway (Villalba *et al*. 1997; Anderson and Tolkovsky 1999; Bonni *et al*. 1999; Mazzoni *et al*. 1999; Han and Holtzman 2000). In this pathway, Raf is recruited to the plasma membrane and directly interacts with GTP-Ras. Upon activation, Raf phosphorylates mitogen-activated protein kinase (MEK), which in turn phosphorylates and activates extracellular signal-regulated kinases (ERK1/2). In neuronal populations in which the Raf-MEK-ERK pathway sustains neuronal survival, ERK activation leads to the activation of the CREB transcription factor or the inactivation of BAD (Bonni *et al*. 1999). Although the ERK pathway is the major effector of Raf, recent evidence suggests that it is not the only one (reviewed in Baccarini 2000; Hindley and Kolch 2002). Mammals possess three Raf proteins: c-Raf (also called Raf-1), A-Raf, and B-Raf (reviewed in Baccarini 2000; Dhillon and Kolch 2002; Hindley and Kolch 2002). While c-Raf is expressed ubiquitously, the expression of B-Raf is restricted primarily to the nervous system. Additionally, B-Raf is the most potent activator of MEK and A-Raf is the weakest. Mice deficient in each of the three Raf genes have been generated. While mice deficient in A-Raf are viable albeit with minor gastrointestinal and neurological defects, disruption of either c-Raf or B-Raf results in embryonic lethality (Wojnowski *et al*. 1997; Huser *et al*. 2001; Mikula *et al*. 2001). Interestingly, sensory neurons and motoneurons cultured from B-Raf-deficient embryos (but not from c-Raf or A-Raf deficient embryos) fail to survive in response to neurotrophic factors (Wiese *et al*. 2001).

The wealth of knowledge that has been recently generated about the molecular regulation of neuronal apoptosis has permitted the rational design of drugs to reduce or prevent neuronal loss in neuropatholgies. Much effort towards development of neuroprotective agents has focused on target molecules such as the JNKs, MLKs, cdks, and the caspases (reviewed in O'hare *et al*. 2002; Saporito *et al*. 2002; Vila and Przedborski 2003). We now show that a c-Raf inhibitor GW5074 {5-Iodo-3-[(3,5-dibromo-4-hydroxyphenyl) methylene]-2-indolinone} is a potent inhibitor of neurodegeneration both in cell culture paradigms and in an animal model. To our knowledge, this is the first demonstration of neuroprotection by pharmacological inhibition of c-Raf. Based on its strong neuroprotective effects on cultured neurons GW5074 could have therapeutic value against neurodegenerative pathologies.

Experimental procedures

Materials

Unless specified otherwise, all chemicals, including GW5074, were purchased from Sigma Chemicals (St. Louis, MO, USA). All antibodies used in this study were purchased from Cell Signaling, Inc. (Beverly, MA, USA) unless specified otherwise. Monoclonal antibodies to c-Raf were purchased from BD Transduction Laboratories (San Jose, CA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA) and a monoclonal antibody to B-Raf was purchased from Santa Cruz Biotechnology. PD98059, *S-trans*, *trans*-farnesylthiosalicyclic acid (FTS) was purchased from Calbiochem (La Jolla, CA, USA), U0126 was purchased from Cell Signaling Technology (Beverly, MA, USA), and ZM336372 was purchased from Tocris (Ellisville, MO, USA). The recombinant adenovirus expressing green fluorescent protein (Ad-GFP) was a gift from Kim A. Heidenreich (University of Colorado Health Sciences Center, Denver, CO, USA), and the adenoviral expression vector encoding a hemagglutinin-tagged (HA) dominant-negative Akt (Ad-dnAkt) was gifted by Wataru Ogawa (Kobe University, Hyogo, Japan). The expression plasmid encoding a kinase-dead GST-MEK1 K97M was a kind gift of Melanie Cobb (University of Texas Southwestern, Dallas, TX, USA).

Cell culture and treatments

Granule neuron cultures were obtained from dissociated cerebella of 7–8 day old rats as described previously (D'Mello *et al*. 1993). Cells were plated in basal Eagle's medium with Earle's salts (BME) supplemented with 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine (Gibco-BRL), and 100 μg/mL gentamycin on dishes coated with poly L-lysine in 24-well dishes at a density 1.0×10^6 cells/well, 1.2×10^7 cells/60 mm dish, or 3.0×10^7 cells/100 mm dish. Cytosine arabinofuranoside (10 μM) was added to the culture medium 18–22 h after plating to prevent replication of non-neuronal cells. Cultures were maintained for 6–7 days prior to experimental treatments. For this, the cells were rinsed once and then maintained in low K^+ medium (serum-free BME medium, 5 mM KCl) or high K^+ medium (serum-free BME medium, supplemented with 20 mM KCl). When used to treat cultures, GW5074 was added at the time

when cells were switched to LK medium. Treatment of cultures with pharmacological inhibitors was initiated 15 min prior to rinsing and was maintained through the subsequent incubation in LK or HK medium unless specified otherwise. For MPP+ and methylmercury treatments, 7–8-day-old cultures were switched to HK medium containing 200 μM MPP+ or 0.5 μM methylmercury. Viability was assayed 24 h later. Glutamate-induced excitotoxicity was performed on 8-day-old cultures. Briefly, culture medium was removed and kept at room temperature. Cells were washed twice with Locke's solution (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 5 mM HEPES, pH 7.4) and incubated at room temperature for 30 min in Locke's solution containing 100 μM glutamate and 1 μM glycine. After treatment, the Locke's solution was removed, the cells washed, and the original culture medium added again. Cell viability was measured 24 h later.

Cortical cultures were obtained from the cerebral cortex of Sprague–Dawley rats (day 17 of gestation) as described previously (Murphy *et al*. 1990). All experiments were initiated 24–72 h after plating. Under these conditions the cells are not susceptible to glutamate-mediated excitotoxicity. For cytotoxicity studies, cells were rinsed with warm phosphate buffered saline (PBS) and then placed in MINIMUM ESSENTIAL Medium (MEM, Gibco BRL) with 5.5 g/L glucose, 10% fetal calf serum, 2 mM L-glutamine, and 100 mM cysteine, containing the glutamate analog, homocysteate (HCA, 1 mM). HCA was diluted from 100-fold concentrated solutions that were adjusted to pH 7.5. To evaluate the effects of GW5074 on HCA-induced cytotoxicity, GW5074 (5 nM–5 μM) was added at the time cortical neurons were exposed to HCA. Viability was assessed 24 h later.

Neuronal survival

Neuronal survival was quantified by the MTT assay as previously described (Koulich *et al*. 2001). Briefly, the tetrazolium salt MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] was added to the cultures at a final concentration of 1 mg/mL, and incubation of the culture was continued in the CO_2 incubator for a further 30 min at 37°C. The assay was stopped by adding lysis buffer [20% sodium dodecyl sulfate (SDS) in 50% *N*,*N*-dimethyl formamide, pH 4.7]. The absorbance was measured spectrophotometrically at 570 nm after an overnight incubation at room temperature. The absorbance of a well without cells was used as background and subtracted. Data are presented as mean ± standard deviation. Statistical analysis was performed using ANOVA and Student–Neuman–Keuls' test. Besides MTT assays, viability was also quantified using the fluorescein-diacetate method (D'Mello *et al*. 1993) and by 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) staining (which reveals apoptotic nuclei as condensed or fragmented). The results using these assays were similar to those obtained with the MTT assay.

Western blotting

For whole-cell lysates, the culture medium was discarded, the neurons washed twice with ice-cold phosphate-buffered saline (PBS), and lysed in lysis buffer [1% Triton, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin and 1× protease inhibitor mixture]. Protein concentrations were measured using a Bradford protein assay kit (Bio-Rad), and equivalent amounts of protein were mixed with 6× SDS sample buffer [375 mM Tris-HCl (pH 6.8 at 25°C), 12% SDS, 60% glycerol, 300 mM dithiothreitol (DTT), 0.012% bromophenol blue]. Following heating at 95°C for 5 min, proteins were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and transferred electrophoretically to polyvinylidene difluoride membrane (Bio-Rad). After staining with Ponceau S to verify uniformity of protein loads/transfer, the membranes were analyzed for immunoreactivity. Incubation with primary antibodies was performed overnight at 4°C and with secondary antibodies for 1 h at room temperature. Immunoreactivity was developed by enhanced chemiluminescence (ECL; Amersham) and visualized by autoradiography.

Immunoprecipitation

After treatment, cultures of 7–8-day-old neurons were washed twice with ice-cold PBS and lysed in lysis buffer. The lysates were centrifuged for 10 min at 10 000 × *g* at 4°C. Protein concentrations of the supernatant were measured using a Bradford protein assay kit (Bio-Rad), and equivalent amounts of protein were incubated overnight with primary antibody (1.0–2 μg) and then for 2 h with 20 μL Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) Immunoprecipitates were collected by centrifugation at 1000 × *g* for 5 min at 4°C and washed three times with lysis buffer, and pellets resuspended in 3× SDS sample buffer, boiled for 4 min and subjected to SDS–PAGE.

In vitro kinase assay

In general, *in vitro* kinase assays were performed using purified kinase and synthetic substrates under standard conditions using the Kinase Profiling service of Upstate Biotechnology. Briefly, for each assay 5–10 mU of purified kinase was used. For GSK3β, cdk1, cdk2, cdk3, cdk5, the kinase was incubated with 1 μM GW5074 in a buffer containing 8 mM MOPS, pH 7.2, 0.2 mM EDTA, 10 mM magnesium acetate and [γ-^{33}P-ATP] for 40 min at room temperature. Kinase activity was quantified by measuring ^{33}P incorporation by spotting an aliquot on P30 filters, washing in 50 mM phosphoric acid and scintillation counting. The buffer composition for c-Raf, JNK1, JNK2, JNK3, MEK1, MKK6, MKK7 was 50 mM Tris pH 7.5, 0.1 mM EGTA, 10 mM magnesium acetate and [γ-^{33}P-ATP]. The peptide substrates used were as follows: For c-Raf, 0.66 mg/mL MBP; for cdks, 0.1 mg/mL histone H1; for JNKs, 3 μM ATF2; for MEK1, 1 μM MAPK2; for MKK6, 1 μM of SAPK2a and for MKK7, 2 μM JNK1α.

Activity of endogenous c-Raf and B-Raf activity was assayed by measuring the ability of kinase immunoprecipitated from neuronal lysates to phosphorylate a kinase-dead recombinant GST-MEK1 K97M substrate. Following immunoprecipitation and multiple washes with lysis buffer, lysis buffer supplemented with 350 mM NaCl, and kinase buffer (25 mM HEPES pH 7.4 and 10 mM $MgCl_2$), *in vitro* kinase assays are performed on the immune complexes using purified recombinant GST-MEK1 K97M protein as a substrate in kinase buffer supplemented with 85 μM ATP for 35 min at 30°C. Reactions are stopped by the addition of 3× SDS sample buffer and boiled for five minutes. Proteins are resolved by SDS–PAGE and subjected to western blotting. The level of phosphorylated MEK is detected by a phospho-MEK antibody.

Overexpression using adenoviral vectors

The hemagglutinin-tagged dominant-negative Akt (Ad-dnAkt) consists of two mutations where the phosphorylation sites at Thr308 and Ser473 are mutated to yield a phosphorylation-deficient inactive protein. Ad-dnAkt and control recombinant adenovirus expressing green fluorescent protein (Ad-GFP) are propagated in HEK293T cells and purified by cesium chloride density gradient centrifugation. After quantification of titer, virus at an MOI of 50 are used to infect granule neuron cultures 5 days after plating by direct addition to the medium. Treatments are performed 24 h after addition of virus. Infected neurons were detected by GFP fluorescence or by positive staining for HA by immunocytochemistry procedures as described previously (Koulich *et al*. 2001). The proportion of apoptotic cells (condensed or fragmented nuclei) as a percentage of total infected neurons was quantified following DAPI-staining.

Gel electrophoresis mobility shift assay

To isolate nuclei, cells were incubated for 30 min in buffer A containing 10 mM HEPES pH 7.9, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 50 mM NaF, 50 mM, α-glycerophosphate, 5% glycerol, and 1 × protease inhibitor cocktail. Following incubation, 1/10 the buffer A volume of 10% NP-40 was added and the mixture was vortexed for 30 s. Cytosolic extracts were obtained following brief centrifugation. The remaining nuclear pellet was resuspended in buffer B containing 20 mM HEPES pH 7.9, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol, 1 × protease inhibitor cocktail and extracted on ice for 30 min, followed by microcentrifugation at 16 000 × *g* for 10 min. The supernatants were collected as nuclear extracts. Concentrations of these nuclear extracts were determined by the Bradford method using reagents from Bio-Rad. Ten micrograms of each nuclear extract sample was incubated with 0.1 pmol of ^{32}P-labeled double-stranded κB binding oligonucleotide (5′-GCTGGGGACTTTC-3′) or SP1 binding oligonucleotide (5′-ATTCGATCGGGGCGGGGCGAGC-3′) in buffer containing 1 μg of poly(dI-dC), 1 μg of bovine serum albumin, 10 mM HEPES pH 7.6, 0.5 mM DTT, 0.1 mM EDTA, 60 mM KCl, 0.2 mM PMSF, 5 mM $MgCl_2$, and 12% glycerol at room temperature for 30 min. Samples were analyzed by 5% native PAGE followed by autoradiography.

3-Nitropropionic acid treatment and experimental design

Eight-week-old C57BL/6 male mice were purchased from Charles River Laboratories, Inc (Wilmington, MA, USA). All experimental protocols were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of Texas at Dallas. 3-NP was dissolved in water and the solution brought to pH 7.4 with sodium hydroxide. 3-NP was administered in 10 intraperitoneal injections (50–55 mg/kg twice a day for 5 days). GW5074 was also administered intraperitoneally at doses of 0.5–10 mg/kg once a day each day 3-NP was administered. Injections of GW5074 were performed 30–60 min before 3-NP administration. Control animals received saline injections. On the day following the 5 days of injection, mice were deeply anesthetized. Brains were removed, washed in PBS, and rapidly frozen in Cryo-Stat embedding medium obtained from StatLab Medical Products, Inc (Lewisville, TX, USA). Coronal sections were cut on a cryostat at 50 microns and stained for Nissl substance with cresyl violet (Sigma).

Behavioral analysis

The Tru-Scan® activity monitoring system (Coulborn Instruments, PA, USA) was used to assess locomotor activity in mice on the day following the 5 days of injection with saline, 3-NP or a combination of 3-NP and GW5074 (7 animals in each group). The animal was placed in a Perspex arena (25.9 × 25.9 cm) with infrared beams spaced at 0.6-inch intervals in the *X–Y* plane. The arena was also equipped with a second infrared beam system at the *Z* plane positioned 2.54 cm above the *X–Y* plane. In this system the movement of the animal is accurately assessed by interruptions in 17 × 17-grid system created by the infrared beams in both the *X–Y* and *Z* planes. The animal was allowed to remain in the arena for 15 min, with data collection performed during this period using the Tru Scan Linc interface box and Tru Scan 99 software, operating through a Pentium PC. The following behavioral parameters were selected: (i) Total movements episodes: each movement in the floor plane is a series of coordinate changes with no rest for at least 1 sample interval; (ii) total movement distance: the sum of all vectored *X–Y* coordinate changes in the floor plane; (iii) mean velocity: the mean velocity of all X-Y coordinate change defined movements; and (iv) vertical plane entries: the total number of times any part of the animal entered the vertical plane (*Z* plane).

Results

GW5074 inhibits LK-induced apoptosis in cerebellar granule neurons

Cultured cerebellar granule neurons undergo apoptosis when switched from HK to medium containing LK (D'Mello *et al*. 1993). Treatment with GW5074 prevents LK-induced apoptosis in these cultures (Fig. 1a,b). Maximal protection against LK-induced apoptosis is observed at 1 μM. Neuroprotection by GW5074 is reduced at 5 μM and is substantially lower at a concentration of ≥ 10 μM. GW5074 has no effect on neuronal survival in HK medium (data not shown).

GW5074 is a potent and specific inhibitor of c-Raf *in vitro*

Lackey *et al.* (2000), reported that GW5074 is a potent inhibitor of c-Raf with no effect on the activities of cdk1, cdk2, c-src, p38 MAP kinase, VEGFR2, and c-fms. We examined the effect of GW5074 on purified c-Raf *in vitro* and confirmed that GW5074 inhibits c-Raf (Table 1).

Although clearly a potent inhibitor of c-Raf, the possibility that GW5074 inhibits other kinases that may have pro-apoptotic effects could not be excluded. A number of reports have implicated JNKs in the promotion of neuronal apoptosis both *in vivo* and in cell culture systems (Coffey *et al*. 2002). Although all three JNK proteins are expressed in neurons, the phosphorylation of c-jun is believed to be mediated by JNK2 or JNK3 (Bruckner *et al*. 2001; Bruckner and Estus 2002; Coffey *et al*. 2002). As shown in Table 1, GW5074 has no direct effect on the activity on any of the three JNK proteins. In apoptotic neurons, JNK activation is mediated primarily

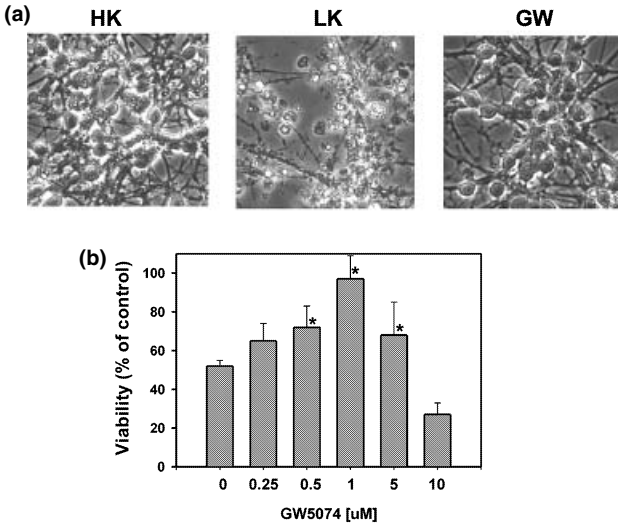

Fig. 1 GW5074 inhibits LK-induced neuronal apoptosis. (a) Phase contrast micrographs showing morphological appearance of neuronal cultures treated with HK, LK or LK + 1 μM GW5074 for 24 h. (b) Quantification of anti-apoptotic effect of GW5074. Neuronal cultures were switched to medium containing LK, or LK medium containing different doses of GW5074. Cell viability was quantified 24 h later. Control cultures received HK medium. Results shown are from three separate experiments. $^*p < 0.001$ mean value ± SD compared with viability of culture receiving LK medium with no GW5074. Similar results were obtained using the fluorescein diacetate viability assay or DAPI staining (not shown).

Table 1 The activity of each kinase was measured *in vitro* the presence 1 μM GW5074. In all cases, ATP was 10 μM. Kinase activity is expressed as a percentage of that in control assays (without GW5074). Results are expressed as mean ± SD

Kinases	% activity
c-Raf	3 ± 0
JNK1	94 ± 3
JNK2	91 ± 4
JNK3	89 ± 9
MEK1	94 ± 4
MKK6	91 ± 4
MKK7	104 ± 8
CDK1/cyclin B	90 ± 12
CDK2/cyclin E	100 ± 5
CDK2/cyclin A	99 ± 0
CDK5/p35	94 ± 2
CDK6/cyclin D3	98 ± 1
GSK3β	81 ± 6

by MKK7 (Eilers *et al*. 1998; Trotter *et al*. 2002). As shown in Table 1, GW5074 has no effect of MKK7 activity. Additionally, GW5074 did not affect MKK6, a kinase that activates p38 MAP kinase, another stress-activated MAP kinase implicated in neuronal apoptosis (Table 1). That GW5074 has no direct effect on p38 MAP kinase itself has previously been reported by Lackey *et al*. (2000).

Several lines of evidence indicate that abortive reentry into the cell cycle by activation of cell cycle components is responsible for apoptosis in granule neurons and other neuronal types (Park *et al*. 1997a, 1997b; Padmanabhan *et al*. 1999). It was thus possible that the neuroprotective effect of GW5074 was mediated by cdk inhibition. As shown in Table 1, however, GW5074 had no effect on the activity of any of the cdks that were examined.

Treatment of neuronal cultures with GW5074 permits accumulation of activating modifications on c-Raf

To examine whether c-Raf was inhibited by GW5074 in granule neurons, culture neurons were treated with GW5074 and the lysates used to immunoprecipitate c-Raf. The immunoprecipitated c-Raf was used in *in vitro* kinase assays using GST-MEK as substrate and the reaction products subjected to western analysis. Phosphorylation of MEK was detected using a phospho-MEK antibody. As shown in Fig. 2(a) c-Raf activity is barely detectable in untreated cultures (maintained in medium containing serum and potassium). This low level of activity remained unchanged in cultures switched to LK medium. Surprisingly, and in apparent contradiction to the results from *in vitro* assays using purified kinase, the activity of c-Raf immunoprecipitated from cultures treated with GW5074 in LK medium displayed a marked induction (Fig. 2a).

c-Raf is inhibited by phosphorylation at Ser259 and activation of c-Raf requires dephosphorylation of this site (Dhillon and Kolch 2002; Hindley and Kolch 2002). We examined the status of Ser259 phosphorylation after GW5074 treatment. When neurons are switched to LK medium, there is a modest but sustained increase in phosphorylation of c-Raf at Ser259, which can be detected within 10 min (Fig. 2b). Although a rapid increase in Ser259 phosphorylation is also seen following treatment with GW5074, this was reduced to a level substantially below basal levels by 30 min of treatment, again suggesting induction of c-Raf activity by GW5074 (Fig. 2b). The dephosphorylation at Ser259 by GW5074 is maintained at 1 and 3 h, while the extent of phosphorylation in LK was similar to that of untreated cultures at these times (not shown).

To study the effect of GW5074 on c-Raf activity further, we immunoprecipitated c-Raf from granule neurons treated with GW5074 but then assayed its activity *in vitro* in the absence or presence of GW5074. While c-Raf immunoprecipitated from GW5074-treated cultures has higher activity relative to control cultures (without GW5074), addition of GW5074 to the immunoprecipitated enzyme *in vitro* inhibits its activity (Fig. 2c) confirming that GW5074 inhibits c-Raf and does so at concentrations which are substantially lower than 1 μM, a dose at which the drug is neuroprotective. Our

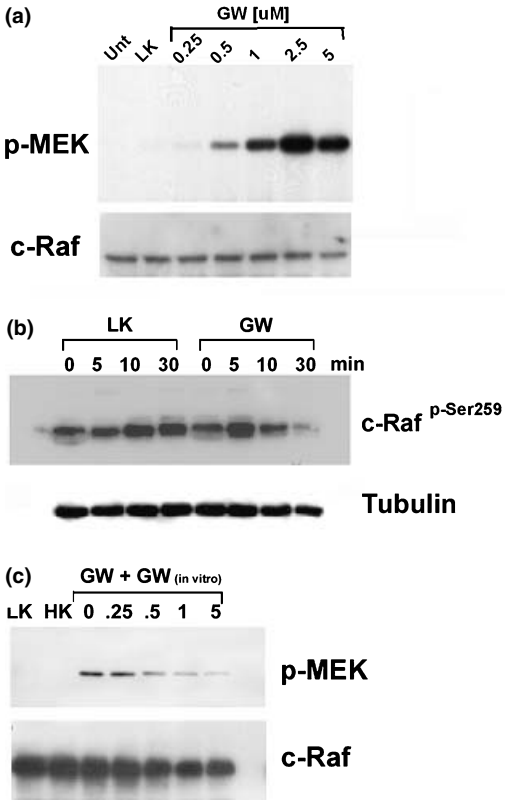

Fig. 2 GW5074 inhibits c-Raf *in vitro* but permits activating modifications to accumulate in intact neurons. (a) *In vitro* kinase assay using MEK substrate. Neurons were either untreated or switched to LK medium containing no additives (LK) or doses of GW5074 (GW) ranging from 0.25 to 5 μM. After 1 h of treatment, the cultures were lysed, and immunoprecipitated c-Raf from the lysate was used in an *in vitro* kinase assay with MEK as substrate. The reaction mixture was subjected to western analysis using a phospho-MEK antibody as probe. The same blot was reprobed with an antibody against c-Raf. Similar results were obtained in two additional experiments. (b) Phosphorylation of c-Raf at Ser259. Neuronal cultures were switched to LK medium containing no additives (LK) or 1 μM GW5074 for 0, 5, 10, and 30 min. The cultures were lysed and the lysate subjected to western blot analysis using a phospho-Ser259–specific antibody. The blot was reprobed with an α-tubulin antibody to show equal loading. The experiment was repeated three times with similar results. (c) Inhibition of c-Raf by GW5074 *in vitro*. Cultures were switched to HK, LK, or LK medium containing 1 μM GW5074 for 1 h. The cultures were lysed, and immunoprecipitated c-Raf from the lysate was used in an *in vitro* kinase assay with MEK as substrate. Aliquots of the immunoprecipitate from the GW5074-treated culture were incubated with different doses of GW5074 ranging from 0 to 5 μM during the *in vitro* kinase reaction. The reaction mixture was subjected to western analysis using a phospho-MEK antibody as probe. The blot was subsequently probed with a c-Raf antibody. The experiment was repeated three times with similar results.

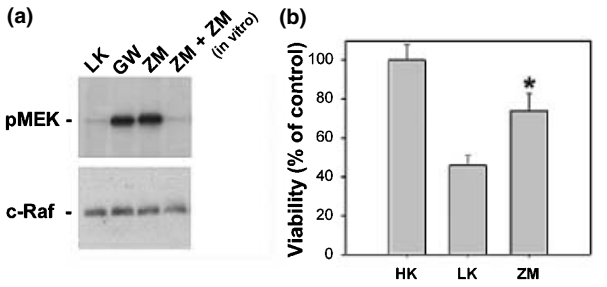

Fig. 3 Effect of ZM336372 on neuronal survival and c-Raf activity. (a) ZM336372 activates c-Raf *in vivo* but is an inhibitor *in vitro*. Neuronal cultures were treated for 1 h with LK, or LK containing 1 μM GW5074 (GW) or 50 μM ZM336372 (ZM) and lysates were obtained. Immunoprecipitated c-Raf from the lysates was used in a kinase reaction with MEK as substrate. In one assay from a ZM336372-treated culture, 10 μM ZM336372 was added to the kinase reaction mixture [ZM + ZM (*in vitro*)]. Phosphorylation of MEK was detected by western blot analysis using a phospho-MEK antibody. The same blot was reprobed with a c-Raf antibody. Similar results were obtained in at least three separate experiments. (b) ZM336372 inhibits LK-induced neuronal apoptosis. Neuronal cultures were switched to HK, LK, or LK medium containing 100 μM ZM336372. Viability was assayed at 24 h using the MTT assay. Results shown are from three separate experiments. $^*p < 0.001$ mean value ± SD compared with viability of culture receiving LK medium with no GW5074. The results were confirmed using DAPI-staining (not shown).

results are consistent with a previously proposed model of c-Raf regulation where c-Raf inhibits its own activity by a mechanism that is as yet unclear (Hall-Jackson *et al*. 1999a; reviewed in Kolch 2000). Inhibition of c-Raf by compounds such as GW5074 could allow activating modifications (such as Ser259 dephosphorylation) to accumulate and result in higher activity if the inhibitor is subsequently removed (as carried out during the process of immunoprecipitation).

ZM336372 is another pharmacological inhibitor of c-Raf that is structurally distinct from GW5074 (Hall-Jackson *et al*. 1999a). While GW5074 inhibits c-Raf with an IC_{50} of 9 nM, ZM336372 is somewhat less potent (IC_{50} = 70 nM; Hall-Jackson *et al*. 1999a). However, like GW5074, ZM336372 treatment causes a paradoxical 'activation' of c-Raf in cell lines. Similarly, immunoprecipitated c-Raf from neuronal cultures treated with ZM336372 has elevated activity, which is inhibited *in vitro* by ZM336372 (Fig. 3a). As shown in Fig. 3(b), ZM336372 inhibits LK-mediated apoptosis, albeit less effectively and at a higher concentration (100 μM) than that of GW5074, consistent with it being a weaker inhibitor of c-Raf. The ability of an independent and structurally distinct pharmacological c-Raf inhibitor to promote neuronal survival suggests that the neuroprotective effect of GW5074 is either directly or indirectly due to its action on c-Raf.

GW5074 activates B-Raf in cultured neurons

Activation of all three Raf proteins results in activation of MEK and ERK. Despite its inhibition of c-Raf, treatment of neuronal cultures with GW5074 leads to an increase in the phosphorylation of ERK 1/2 (Fig. 5a) suggesting that

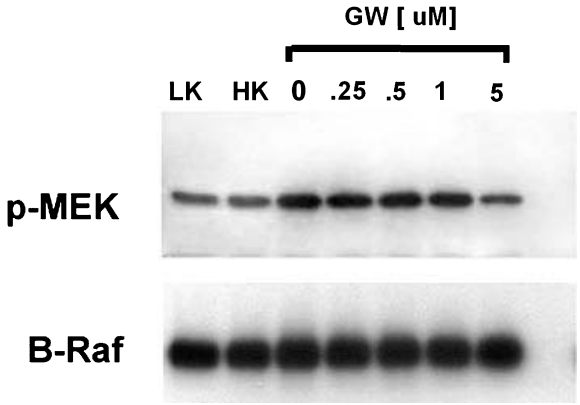

Fig. 4 GW5074 activates B-Raf in neurons leading to ERK phosphorylation. *In vitro* kinase assay for B-Raf. Neuronal cultures were switched to medium containing HK or LK medium containing no additives (LK) or 1 μM GW5074 (GW). After 1 h of treatment, cultures were lysed, and B-Raf immunoprecipitate from the lysates were used in *in vitro* kinase assays with MEK as substrate. Aliquots of the immunoprecipitate from the GW5074-treated culture were incubated with different doses of GW5074 ranging from 0.25 to 5 μM during the *in vitro* kinase reaction. The reaction mixture was subjected to western analysis using a phospho-MEK antibody as probe. The same blot was reprobed with an antibody to B-Raf. Similar results were obtained when the experiment was repeated two additional times.

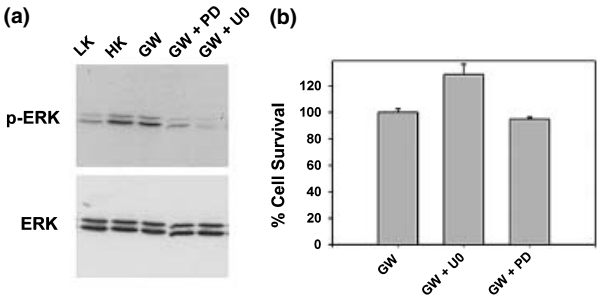

Fig. 5 GW5074-mediated neuroprotection is MEK-ERK independent. (a) Neuronal cultures were treated for 1 h with HK, LK, or LK medium containing 1 μM GW5074 (GW) in the absence or presence of 40 μM PD98059 (GW + PD) or 10 μM U0126 (GW + U0). Lysates from the cultures were subjected to western blotting using an antibody specific for phospho-ERK. The same blot was reprobed with an antibody against total ERK (lower panel). The experiment was repeated twice with similar results. (b) Neuronal cultures were switched to LK or LK medium with 1 μM GW5074 in the absence (GW) or presence of 40 μM PD98059 (GW + PD) or 10 μM U0126 (GW + U0). Cell viability was quantified 24 h later and expressed as percentage of viability in control cultures that received HK medium. The results shown come from three independent experiments.

GW5074 treatment caused the activation of either A-Raf or B-Raf. As B-Raf is highly expressed in the nervous system and as B-Raf rather than c-Raf is the major stimulator of ERK (Marais *et al*. 1997; York *et al*. 1998), we examined the effect of GW5074 on B-Raf. As shown in Fig. 4, cerebellar granule neurons have clearly detectable basal B-Raf activity. B-Raf activity is elevated further by treatment with GW5074.

To examine if like c-Raf, B-Raf was also inhibited by GW5074 *in vitro*, we immunoprecipitated B-Raf and examined the effect of the drug on its activity. In contrast to c-Raf, GW5074 had no effect on B-Raf activity when used at concentrations of up to 1 μM (Fig. 4). GW5074 did inhibit B-Raf at 5 μM, a concentration at which the protective effect of the drug on LK-induced apoptosis is somewhat reduced (see Fig. 1).

Neuroprotection by GW5074 is MEK-ERK independent

Survival of cerebellar granule neurons can be maintained by BDNF. This effect of BDNF is mediated by the Raf-MEK-ERK signaling pathway (Bonni *et al*. 1999). The Raf-MEK-ERK pathway is also involved in promoting survival of other neuronal and non-neuronal cell types. A potent blocker of the Raf-MEK-ERK pathway is PD98059 (Allessi *et al*. 1995). In paradigms in which the Raf-MEK-ERK pathway mediates neuronal survival, such as BDNF-treated cerebellar granule neurons, the presence of PD98059 blocks survival (Bonni *et al*. 1999). As shown in Fig. 5(a), treatment with PD98059 blocks the stimulation of ERK by GW5074. This, however, had no effect on the neuroprotective effect of GW5074 (Fig. 5b). U0126, a structurally independent MEK inhibitor, which potently inhibits both MEK1 and MEK2 (Duncia *et al*. 1998; Favata *et al*. 1998; Fig. 5(a) also failed to reduce survival by GW5074 (Fig. 5a,b). These results show that neuroprotection by GW5074 is MEK-ERK independent.

GW5074 delays down-regulation of Akt activity but inhibits apoptosis by an Akt-independent mechanism

The best-studied anti-apoptotic pathway in neurons is the PI-3K-Akt signaling pathway (D'Mello *et al*. 1997; Datta *et al*. 1997; Dudek *et al*. 1997; Miller *et al*. 1999). Insulin-like growth factor (IGF-1), a potent survival-promoting factor for granule neurons mediates its effect by activating the PI-3K-Akt pathway (D'Mello *et al*. 1997; Dudek *et al*. 1997). As the Raf-MEK-ERK pathway was not necessary for the neuroprotective action of GW5074, we examined the possibility that GW5074 exerted its protective effect by engaging this pathway. Cross-talk between Raf/Ras and Akt has been demonstrated by a number of laboratories (reviewed in Jun *et al*. 1999). As we have reported, switching cerebellar granule neurons from serum-containing HK medium in which they are cultured and allowed to mature, to serum-free LK or HK medium, causes a rapid down-regulation of Akt phosphorylation and activity, which can be prevented by IGF-1 (Kumari *et al*. 2001). As shown in Fig. 6(a), GW5074 delays the down-regulation of Akt phosphorylation observed after LK treatment. GSK3β is a pro-apoptotic molecule that is activated during apoptosis in many neuronal and non-neuronal systems. Under survival promoting conditions

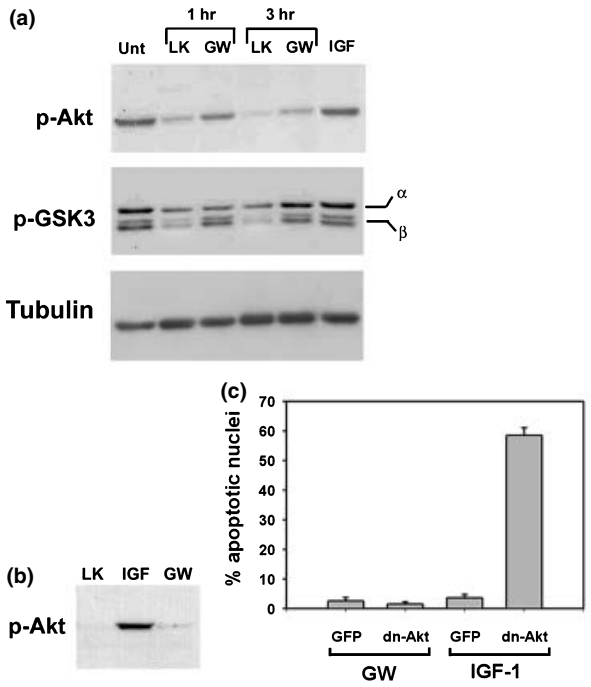

Fig. 6 GW5074 maintains Akt activity but acts through an Akt-independent mechanism. (a) Lysates from neurons treated for 1 h and 3 h with LK or LK medium containing 1 μM GW5074 (GW) were subjected to western blotting using an antibody against phospho-Akt (Ser473). The same blot was reprobed with antibodies against phospho-Gsk3α/β and with α-tubulin. Also loaded on the gel were lysates from untreated cultures (maintained in medium containing serum and high K +) and a culture treated for 1 h with 25 ng/mL IGF-1. The results were reproduced in two other experiments. (b) Neuronal cultures were switched to LK medium for 2 h to down-regulate basal Akt activity. The medium was then supplemented with no additives (LK), 25 ng/mL IGF-1, or 1 μM GW5074 (GW) for 30 min. The lysates from these cultures were used for western blotting with an antibody for phospho-Akt (Ser473). The results shown are representative of three experiments. (c) Five-day-old neuronal cultures were infected with adenoviral vectors expressing either GFP or hemaglutinin (HA)-tagged dominant-negative Akt. The next day the cultures were switched to LK medium containing 1 μM GW5074 (GW) or 25 ng/mL IGF-1 (IGF). Infected neurons were detected by positive staining for GFP or HA by immunocytochemistry. The proportion of apoptotic cells (condensed or fragmented nuclei) as a percentage of total infected neurons was quantified following DAPI-staining.

GSK3β is kept inactivated by phosphorylation, a modification that can be induced by Akt. As shown in Fig. 6(a), GW5074 prevents the activation of GSK3β that occurs after the switch to LK medium. Another pro-apoptotic molecule that is phosphorylated and inactivated by Akt is the transcription factor, Forkhead (Brunet *et al*. 1999). GW5074 reduces the dephosphorylation of Forkhead that is observed in LK (data not shown). Switching of neurons to LK medium leads to a rapid down-regulation of Akt activity within 2 h. Addition of GW5074 to such cultures does not lead to Akt activation. In contrast, addition of IGF-1 causes a robust increase in Akt phosphorylation (Fig. 6b). Thus, while capable of temporarily maintaining the activity of Akt in LK, treatment with GW5074 cannot activate Akt *de novo*.

To determine if Akt activity was necessary for the neuroprotective action of GW5074, we treated neurons with GW5074 after infecting them with an adenoviral vector expressing a dominant-negative form of Akt. While blockade of Akt activity using this approach reduces the survival-promoting effect of IGF-1, it had no effect on the ability of GW5074 to maintain neuronal survival (Fig. 6c). This result indicates that although maintaining Akt activity in LK, the neuroprotective action of GW5074 is mediated by an Akt-independent mechanism.

GW5074 affects Ras, nuclear factor-kappa B (NF-κB) and c-jun

Activation of c-Raf and B-Raf is often mediated by Ras. Blockade of c-Raf signaling by GW5074 could lead to an accumulation of activated Ras, which could lead to the stimulation of an alternative, anti-apoptotic pathway. While known to exert anti-apoptotic effects by a PI-3 kinase-Akt dependent pathway, Ras has also been shown to provide an anti-apoptotic signal through an Akt-independent mechanism involving down-regulation of JNK and p38 activity (Wolfman *et al*. 2002). To determine if Ras was necessary for GW5074-mediated neuroprotection, we used *S-trans*,*trans*-farnesylthiosalicylic acid (FTS). FTS is a cell permeable Ras antagonist that dislodges Ras from its membrane-anchoring sites leading to its degradation and thus causing a decrease in total cellular Ras (Jansen *et al*. 1999). As shown in Fig. 7(a), treatment with FTS blocks the neuroprotective effect of GW5074.

Another molecule known to be important for neuronal survival is NF-κB. Activation of NF-κB protects neurons against a variety of apoptotic stimuli and reduced NF-κB activity has been associated with neurodegeneration both *in vitro* and *in vivo* survival (Yu *et al*. 1999; Glazner *et al*. 2000; Koulich *et al*. 2001; Yabe *et al*. 2001; Bhakar *et al*. 2002; reviewed in Mattson *et al*. 2000). Although the precise mechanism has not been elucidated, c-Raf is known to activate NF-κB in non-neuronal cells via a MEK-ERK-independent mechanism (Foo and Nolan 1999; Pearson *et al*. 2000). Because of the established role of NF-κB in neuronal survival and the existence of a signaling pathway connecting Raf to NF-κB, we considered whether neuroprotection by GW5074 involved NF-κB. We first examined the effect of GW5074 treatment on the DNA-binding activity of NF-κB. As shown in Fig. 7(b), and as we have previously reported, treatment of cerebellar neuron cultures with LK leads to a down-regulation of NF-κB activity. This down-regulation of NF-κB binding activity is prevented by GW5074 (Fig. 7b). We next examined if NF-κB was required for the neuroprotective effect of GW5074. As

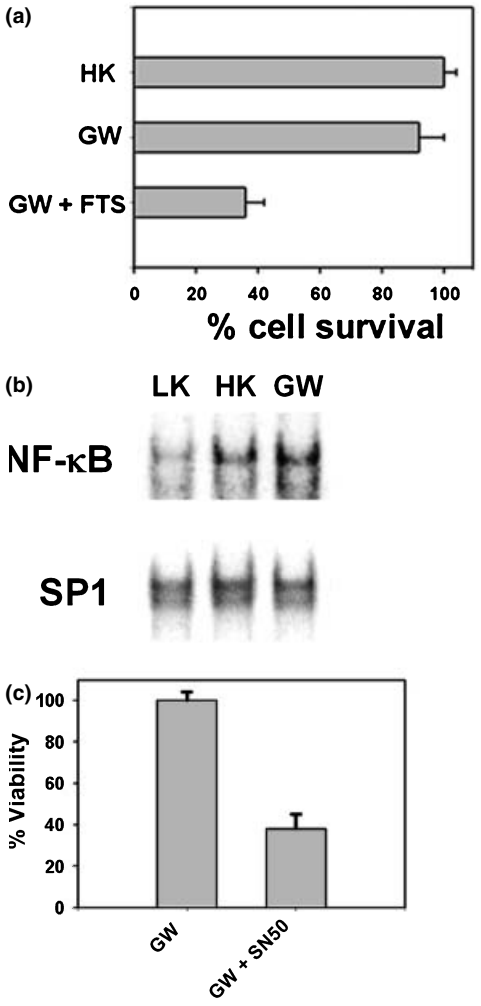

Fig. 7 Neuroprotection by GW5074 requires Ras and NF-κB. (a) Neuronal cultures were switched to medium containing HK or to LK medium containing 1 μM GW5074 in the absence (GW) or presence of 10 μM FTS (GW + FTS). Viability was quantified 24 h later and expressed as percentage of viability in HK. $^*p < 0.001$ mean value ± SD compared with viability of culture receiving GW. The results shown come from three independent experiments. (b) Nuclear extracts from cultures treated for 6 h with HK, LK, or LK medium containing 1 μM GW5074 (LK + GW) were used in gel mobility shift assays with radioactively labeled oligonucleotide probes containing the NF-κB binding site or the SP1 binding site. (c) Neuronal cultures were switched to LK medium containing 1 μM GW5074 in the absence (GW) or presence of 10 μM SN-50 (GW + SN-50). Viability was quantified 24 h later. $^*p < 0.001$ mean value ± SD compared with viability of culture receiving GW. The results are from three independent experiments.

shown in Fig. 7(c), treatment with SN-50, a synthetic cell-permeable peptide that has been demonstrated to potently and specifically inhibit NF-κB activity, blocks the ability of GW5074 to inhibit LK-induced apoptosis indicating that the neuroprotective effect of GW5074 is NF-κB-dependent.

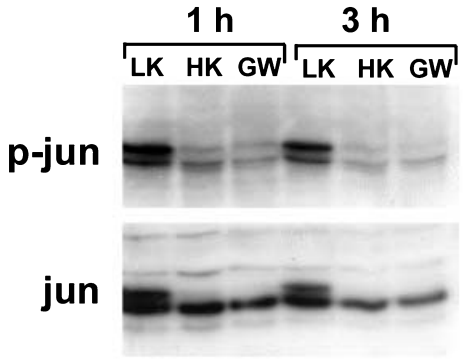

Fig. 8 GW5074 inhibits apoptosis-associated induction of c-jun. Lysates from neurons treated for 1 and 3 h with HK, LK or LK medium containing 1 μm GW5074 (GW) were subjected to western blotting using an antibody against phospho-c-jun (Ser63). The same blot was reprobed with an antibody against total c-jun (lower panel). Similar results were obtained in at least three independent experiments.

Phosphorylation of c-jun is necessary for neuronal apoptosis in a variety of paradigms including LK-induced death of granule neurons (Estus *et al*. 1994; Ham *et al*. 1995; Watson *et al*. 1998). In granule neurons, phosphorylation of c-jun occurs within 1 h of LK treatment (Ham *et al*. 1995; Watson *et al*. 1998). As shown in Fig. 8, GW5074 treatment prevents LK-induced c-jun phosphorylation and its increased synthesis.

GW5074 inhibits cell death caused by neurotoxins in granule cells and other neuronal types

1-Methyl-4-phenyl-1,2,3,6-tetrathydropyridine is a neurotoxin that causes degeneration of nigrostratial dopaminergic neurons in humans and some experimental animals resulting in a Parkinson's like pathology. The neurotoxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrathydropyridine are mediated through its oxidation by astrocytes to the neurotoxic species 1-methyl-4-phenylpyridinium (MPP+) that is taken up actively by dopaminergic neurons through the dopamine transporter. Direct treatment of dopaminergic neurons in culture with MPP+ recapitulates their degeneration seen *in vivo*. In culture, MPP+ is also toxic to cerebellar granule neurons (Gonzalez-Polo *et al*. 2001). Another neurotoxic agent that causes selective loss of cerebellar granule neurons *in vivo* and which induces apoptosis in cultured cerebellar granule neurons is methylmercury (Kunimoto 1994). As a step towards determining whether GW5074 is protective against other neurotoxic stimuli we examined its ability to prevent cell death in granule neuron cultures treated with MPP+ or methylmercury. As shown in Fig. 9(a,b), GW5074 reduced MPP+ and methylmercury-induced cell death substantially when used at 0.5 and 0.12 μM, respectively. Treatment of cultured cortical neurons with the glutamate analog HCA causes oxidative stress due to glutathione depletion leading to apoptosis (Murphy *et al*. 1990; Ratan

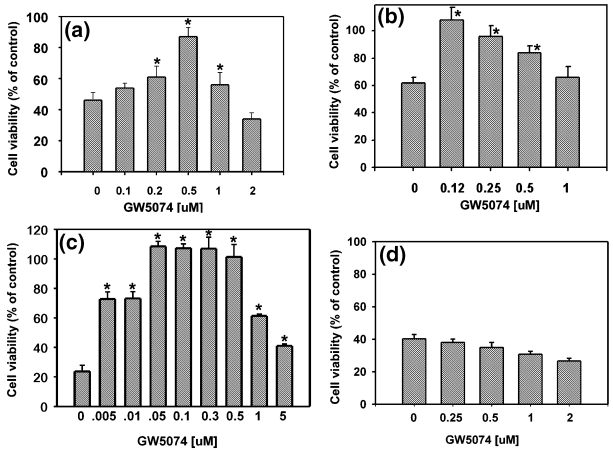

Fig. 9 GW5074 is protective against apoptosis induced by MPP+, methylmercury, and oxidative stress. The ability of different doses of GW5074 to protect against apoptosis induced by various stimuli was tested in cultured cerebellar granule neurons (a,b,d) and in cortical neurons (c). Viability was measured 24 h later. Results shown are from three separate experiments. $^*p < 0.001$ mean value ± SD compared with viability of culture with apoptotic stimulus but no GW5074. (a) Cerebellar granule neurons were treated with HK medium or HK medium containing 200 μM MPP+ in the presence of different concentrations of GW5074 (GW). (b) Cerebellar granule neurons were treated with HK medium or HK medium containing 0.5 μM methylmercury in the presence of different concentrations of GW5074 (GW). (c) Cortical neurons were treated with HCA and different concentrations of GW5074. (d) Cerebellar granule neurons were treated with 100 μM glutamate and 1 μM glycine in Locke's solution for 30 min. The original culture medium was then put back and the cells maintained in the incubator for 24 h.

et al. 1994a,b). To determine whether the effects of GW5074 extended to neuronal types other than cerebellar granule cells, we utilized this *in vitro* paradigm. As shown in Fig. 9(c), GW5074 provided complete protection against oxidative stress-induced apoptosis. Substantial protection is observed at doses as low as 5 nM of GW5074. It is likely that the difference in doses at which GW5074 exerts its neuroprotective effect in the various paradigms of neuronal cell death is due to differences in the intracellular molecular composition between neuronal cell types and in the activities of signaling molecules following treatment with different apoptotic stimuli. For example, while LK treatment down-regulates Akt activity (Dudek *et al*. 1997; Kumari *et al*. 2001), methylmercury-induced neurotoxicity in cerebellar granule neurons is accompanied by Akt activation (Manlapaz and D'Mello, unpublished observation). It is thus possible that Akt assists in GW5074-mediated protection against methylmercury toxicity, requiring a lower concentration of GW5074 for neuroprotection.

Although highly protective against the stimuli described above, GW5074 was unable to reduce glutamate-induced excitotoxicity in cultured cerebellar granule neurons (Fig. 9d). Previous studies have suggested that glutamate-induced neurotoxicity in cerebellar granule neurons does not involve an apoptotic mechanism (Dessi *et al*. 1993).

GW5074 is protective in an *in vivo* experimental model of Huntington's disease (HD)

3-Nitropropionic acid (3-NP) administration in rodents and non-human primates has served as a useful experimental model for HD (reviewed in Brouillet *et al*. 1999). 3-NP is an irreversible inhibitor of succinate dehydrogenase (complex II), which causes prolonged mitochondrial energy impairment and replicates most of the clinical and pathophysiological hallmarks of HD including selective striatal degeneration, spontaneous choreiform and dystonic movements (Beal *et al*. 1993; Brouillet *et al*. 1999). We tested whether GW5074 could protect against 3-NP-induced neurodegeneration. As shown in Fig. 10 (top panel), mice administered 3-NP display extensive bilateral striatal lesions. This degeneration is completely prevented by GW5074 when administered at a concentration of 5 mg/kg body weight. Administration of GW5074 alone had no discernible effect on the animals as judged by behavior and cell morphology of brain sections (data not shown).

The protection by GW5074 against 3-NP-induced striatal neurodegeneration correlated with a robust protection against neurological deficits. Figure 11 shows the analysis of the

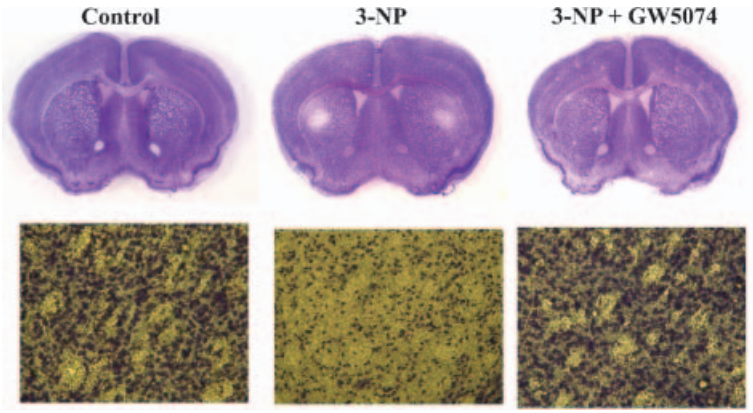

Fig. 10 3-NP induced striatal degeneration is prevented by GW5074. Cresyl violet staining of 50 μm coronal sections from control, 3-NP, and 3-NP + GW5074-treated mice. Doses and conditions of administration are detailed in Experimental procedures. (Top panel) Low magnification showing selective loss of cells in the striatum. (Bottom panel) High magnification image of dorsolateral part of the striatum.

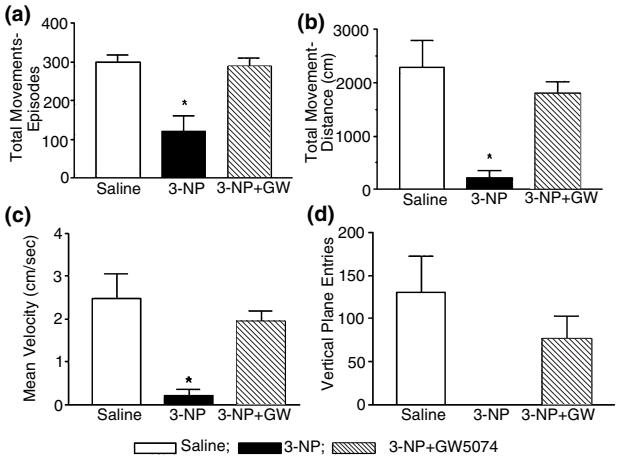

Fig. 11 GW5074 protects against 3-NP-induced behavioral abnormalities. Locomotor activity measurements of mice administered with saline (control), 3-NP and 3-NP + GW5074. Doses and conditions of administration are detailed in Experimental procedures. Activity was measured over a 15-min period. Shown are: (a) total movement episodes; (b) total movement distance; (c) mean velocity; (d) vertical plane entries. Bars indicate mean ± SD (seven animals in each group). *$p < 0.001$; Dunnett's multiple comparison *t*-test versus saline group.

parameters used to assess locomotor activity. For each of the 4 parameters studied, 3-NP treatment resulted in a significant reduction of locomotor activity. Combinatorial treatment with 3-NP and GW5074 restored locomotor activity to the same level as that seen in saline treated animals. Specifically, total movement episodes, total movement distance; mean velocity of movement and vertical plane entries (ability of the animal to rear itself on its hind legs), which were all impaired by 3-NP administration, were restored in animals receiving GW5074.

Discussion

Neurological diseases disrupt the quality of the lives of patients and often lead to their death prematurely. A common feature of most neurological diseases is the degeneration of neurons, which results from an inappropriate activation of apoptosis. Drugs that inhibit neuronal apoptosis could thus be candidates for therapeutic intervention in neurodegenerative disorders. In this report, we describe that GW5074, a commercially available and small molecule chemical compound has strong neuroprotective effects. We show that GW5074 blocks apoptosis in cerebellar granule neurons induced by diverse stimuli such as LK-treatment or exposure to MPP+ or methylmercury. The drug also prevents oxidative stress-induced apoptosis in cortical neurons. Most significantly, GW5074 prevents neurodegeneration and improves behavioral outcome in an *in vivo* experimental model of Huntington's disease.

Based on a previous report (Lackey *et al*. 2000) and our own findings, GW5074 is a potent inhibitor of c-Raf. Lackey *et al*. (2000), showed that GW5074 had no significant effect on cdk1, cdk2, c-src, p38 MAP kinase, VEGFR2, and c-fms. We have confirmed and extended their analysis to show that GW5074 does not inhibit JNK1, JNK2, JNK3, MEK1, MKK6, MKK7, cdk5, cdk6 and Gsk3β. Like GW5074, ZM336372, which is a structurally distinct inhibitor of c-Raf, is also significantly protective against LK-induced neuronal apoptosis. The ability of an independent pharmacological c-Raf inhibitor to promote neuronal survival suggests that the neuroprotective effect of GW5074 is either directly or indirectly due to its action on c-Raf. Another agent that is protective against LK-induced apoptosis is SB203580 (Yamagishi *et al*. 2001; Coffey *et al*. 2002), best known for its inhibition of p38 MAP kinase signaling. It has recently been discovered that SB203580 also inhibits c-Raf potently when assayed *in vitro* (Hall-Jackson *et al*. 1999b) raising the possibility that its ability to inhibit c-Raf may contribute to its ability to protect against apoptosis in granule neurons and other neuronal types.

Although inhibiting c-Raf potently, the treatment of neuronal cultures with GW5074 causes the accumulation of activating modifications on c-Raf. A similar accumulation of activating modifications has also been reported to occur in cell lines treated with other c-Raf inhibitors such as ZM336372 or SB203580 (Hall-Jackson *et al*. 1999a). As previously proposed to occur in cell lines (Hall-Jackson *et al*. 1999a), upon exposure to c-Raf inhibitors, neurons may respond by activating compensatory mechanisms in an attempt to maintain total Raf activity. Besides the accumulation of activating modifications on c-Raf, this may also shunt Ras signaling to B-Raf. Indeed, treatment with GW5074 does lead to the activation of B-Raf. The finding that Ras inhibition blocks neuroprotection by GW5074 is consistent with the redirection of Ras-Raf-MEK-ERK signaling via B-Raf. It is possible that the activation of B-Raf is necessary for GW5074-mediated neuroprotection. Consistent with the involvement of B-Raf is our observation that in contrast to c-Raf, the activity of B-Raf is not inhibited by GW5074 at concentrations at which the drug is neuroprotective. When used at a concentration at which B-Raf is inhibited however, GW5074 is not protective. B-Raf is required for the survival of embryonic sensory and motoneurons and neurons cultured from B-Raf knockout mice die even in the presence of neurotrophic factors that are normally capable of promoting their survival (Wiese *et al*. 2001). Interestingly, overexpression of c-Raf fails to rescue B-Raf–/– neurons from death in culture indicating a critical and essential role for B-Raf in neuronal survival (Wiese *et al*. 2001). B-Raf activation was also been shown to inhibit growth factor induced apoptosis in fibroblasts (Erhardt *et al*. 1999). In this case B-Raf was found to act at a step downstream of cytochrome c release.

Treatment with GW5074 results in the activation of the Raf-MEK-ERK pathway. The Raf-MEK-ERK pathway is known to be important for neuronal survival by some neurotrophic factors. In cerebellar granule neurons, BDNF-mediated survival is mediated by a Raf-MEK-ERK-dependent mechanism resulting in the activation of CREB and the inactivation of the pro-apoptotic Bcl-2 protein, Bad (Bonni *et al*. 1999). Treatment with the MEK inhibitor, PD98059, blocks the survival effect of BDNF on granule neurons (Bonni *et al*. 1999). We find that neither PD98059 nor another potent MEK inhibitor U0126, reduce the ability of GW5074 to protect against LK-induced neuronal death although both of these inhibitors block ERK stimulation, indicating that neuroprotection by GW5074 is mediated by a MEK-ERK-independent signaling pathway. Evidence for the presence of such a Raf-mediated but MEK-ERK-independent anti-apoptotic pathway has been reported by a number of laboratories (reviewed in Baccarini 2000; Dhillon and Kolch 2002; Hindley and Kolch 2002). One potential mediator of MEK-ERK-independent B-Raf signaling is NF-κB. Several studies performed in a variety of *in vivo* and *in vitro* paradigms have shown the importance of NF-κB for neuronal survival (Yu *et al*. 1999; Glazner *et al*. 2000; Koulich *et al*. 2001; Yabe *et al*. 2001; Bhakar *et al*. 2002; reviewed in Mattson *et al*. 2000). The ability of c-Raf to signal via NF-κB in a MEK-ERK independent manner has been documented (Baumann *et al*. 2000). In cerebellar granule neurons, NF-κB is required for the survival promoting effect of diverse survival factors (Koulich *et al*. 2001). A downstream event in GW5074-mediated neuroprotection is likely to be inhibition of c-jun, a molecule whose activation is required for LK-induced neuronal cell death.

Given the established importance of the PI-3 kinase Akt pathway in the inhibition of apoptosis we examined whether GW5074 acted by stimulating this pathway. Treatment with GW5074 does delay the down-regulation of Akt phosphorylation that occurs in LK. Consistent with the maintenance of Akt activity, the dephosphorylation of GSK3-β and Forkhead that occurs after LK treatment, is reduced by GW5074. Inhibition of Akt by overexpression of a dominant-negative form of the protein does not, however, reduce the neuroprotective effect of GW5074 indicating that Akt activity is not required for the neuroprotective effect of GW5074.

The PI-3k-Akt and the Raf-MEK-ERK pathways are the two best studied survival-promoting signaling mechanisms in neurons and non-neuronal cells. Our results indicate that GW5074 acts by a mechanism that is independent of both these pathways suggesting the existence of another potent signaling pathway by which the survival of neurons can be mediated. While additional research is required to elucidate the components of this signaling pathway our results are consistent with a model in which c-Raf inhibition by GW5074 causes induction of B-Raf activity which acts via NF-κB to inhibit c-jun. It is possible that induction of B-Raf depends on Ras.

Neuronal loss by apoptosis is observed in a number of neurodegenerative diseases. Based on its neuroprotective effect against different apoptosis-inducing stimuli *in vitro* and in an animal model of neurodegeneration, inhibitors such as GW5074 could have therapeutic value in the treatment of neurodegenerative diseases. Furthermore, understanding the signaling pathway by which GW5074 exerts its effect could lead to the development of novel and effective drugs for the treatment of neurodegenerative conditions.

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A patent application to the U.S. Patent and Trademark Office for the use of c-Raf inhibitors including GW5074 for the treatment of neurodegenerative diseases has been filed through the University of Texas at Dallas.

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p21-Activated Kinase-1 Is Necessary for Depolarization-Mediated Neuronal Survival

Kyle Johnson and Santosh R. D'Mello*
Department of Molecular and Cell Biology, University of Texas at Dallas, Richardson, Texas

Cerebellar granule neurons undergo apoptosis when switched from culture medium containing high potassium (HK) to medium that contains low potassium (LK). HK treatment leads to an activation of p21-activated kinase-1 (PAK-1). Overexpression of a constitutively active form of PAK-1 protects against apoptosis in LK medium. Overexpression of a dominant-negative form of PAK-1 blocks survival in HK. Although PAK-1 is usually considered to be a downstream effector of Rac and Cdc42, we were unable to detect association between PAK-1 and either Rac1 or Cdc42 in cerebellar granule neurons. Interaction between PAK-1 and PDK1 is detected in granule neurons, although there is no change in the extent of interaction in neurons primed to die. Neuronal survival by PAK-1 overexpression is not inhibited by PD98059 or LY294002, which inhibit the activity of MEK and PI-3 kinase, respectively. The ability of PAK-1 to maintain neuronal survival is, however, blocked by ML-9, a compound known to inhibit Akt. Our results show that that PAK-1 is necessary for neuronal survival in HK and suggest that its neuroprotective action may be mediated by a GTPase-independent, but Akt-dependent, mechanism.

Key words: PAK-1; cerebellar granule neurons; apoptosis; neurodegeneration; potassium; Rho GTPases

Elimination of superfluous neurons by apoptosis is a critical aspect of normal neurodevelopment. Aberrant activation of apoptosis in the mature brain, however, leads to undesirable neuronal loss and has been implicated in a variety of neurodegenerative diseases. How apoptosis is regulated in neurons has thus become an area of intense investigation. Such studies have led to the identification of several molecules that are involved in promoting or inhibiting neuronal cell death. Among these are the Rho-related GTPases (Rho, Rac, and Cdc42), best known for their role in regulating cytoskeletal dynamics (for review see Nobes and Hall, 1995). Recent studies have shown that, depending on the neuronal type, Rho GTPases may either be proapoptotic or antiapoptotic. In sympathetic neurons, for example, inhibition of Rac and Cdc42 by overexpression of dominant-negative forms of these proteins prevents apoptosis induced by nerve growth factor withdrawal (Bazenet et al., 1998; Kanamoto et al., 2000). On the other hand, pharmacological inhibition of Rho GTPases induces apoptosis in cultures of cortical neurons (Tanaka et al., 2000). Similarly, treatment of cultured cerebellar granule neurons with *Clostridium difficile* toxin B, a specific inhibitor of Rho GTPases, blocks serum- and depolarization-dependent survival (Linseman et al., 2001). Although the molecular basis for the opposing effect of Rho GTPases on the survival of different neuronal types is unclear, one explanation might lie in the ability of Rho GTPases to engage different downstream effector molecules.

One class of mediators of Rac and Cdc42 GTPase activity is the p21-activated kinases (PAKs), a family of serine-threonine kinases that are activated in response to extracellular signals (for review see Jaffer and Chernoff, 2002; Bokoch, 2003). Although first identified as proteins that play an important role in regulating cytoskeletal organization and cell morphology, the PAKs have also been shown to regulate cell survival. Six mammalian PAKs have been identified, and these have been classified into two subfamilies based on structural similarity. Group A PAKs consist of the closely related PAK-1, PAK-2, and PAK-3. PAK-1 and PAK-3 are tissue specific, with the highest expression level in the brain, whereas PAK-2 is ubiquitous (Jaffer and Chernoff, 2002; Bokoch, 2003). PAKs 4–6 belong to the group B family. All of the PAKs contain a GTPase binding domain, which binds to the Rho GTPases, and a kinase domain, which shares similarity to the kinase domain of yeast STE20. The group A and B PAKs are ~50% identical to each other in the GTPase binding domain and kinase domain but otherwise share no homology in the rest of their amino acid sequences. Despite this, both group A and group B PAKs have been implicated in the regulation of cell survival. PAK-1 and PAK-4 have been shown to protect cells from apoptosis in a variety of cell types (Shurmann et al., 2000; Gnesutta et al., 2001). In contrast, PAK-2 can be cleaved by caspases into a 34-kDa activated form that contributes to the morphologic changes that occur during apoptosis (Lee et al., 1997;

*Correspondence to: Santosh R. D'Mello, Department of Molecular and Cell Biology, University of Texas at Dallas, 2601 N. Floyd Road, Richardson, TX 75083. E-mail: dmello@utdallas.edu

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Rudel and Bockoch, 1997; Walter et al., 1998). The residue on PAK-2 that is cleaved by caspase-3 (Asp212) is absent in PAK-3 and inaccessible in PAK-1, explaining why these PAKs are not cleaved.

Although PAKs have been found to regulate the survival of nonneuronal cells in response to certain stimuli, the role of PAKs in the regulation of neuronal survival has not been investigated. Here we have studied this issue by using cultured cerebellar granule neurons. Because of its high expression in the brain, we focused on PAK-1. We report that PAK-1 is activated by membrane depolarization as well as by insulin-like growth factor-1 (IGF-1), two stimuli that sustain the survival of these neurons in culture. By using active and dominant-negative forms of PAK-1, we show that PAK-1 plays an important role in promoting neuronal survival.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all chemicals came from Sigma-Aldrich (St. Louis, MO). Cell culture supplies were purchased from Gibco BRL (Grand Island, NY). PD98059, ML-9, and LY294002 were from Calbiochem (La Jolla, CA). Anti-PAK-1 (sc-882), anti-PAK-2 (sc-7117), anti-Rac1 (sc-217), and anti-Cdc42 (sc-8401) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PDK1 (3062) and anti-β-galactosidase (14B7) were purchased from Cell Signaling (Beverly, MA). Anti-α-tubulin (T5168) was purchased from Sigma-Aldrich. IGF-1 was purchased from Roche (Indianapolis, IN).

Cell Culture and Treatment

Culture of cerebellar granule neurons was performed as previously described (D'Mello et al., 1993). Briefly, cerebella were extracted from 7–8-day-old Wistar rat pups and plated in basal medium with Earl's salts (BME), 25 mM potassium, 100 μg/ml gentamicin, 2 mM glutamine, and 10% fetal calf serum (Gibco BRL, Carlsbad, CA) on poly-L-lysine-coated plates. Cells were plated at density of 1×10^6/well in four-well dishes and 1×10^7 cells per 60-mm dish. Cytosine arabinofuranoside at 10 μM was added to the culture medium 18–22 hr after plating to prevent replication of nonneuronal cells. Treatments were performed 6–7 days after plating. For treatments, the cultures were switched to serum-free BME medium in the absence (LK medium) or presence (HK medium) of 25 mM KCl. When used, pharmacological inhibitors were added at the time of the switch. In some experiments (indicated in the figure legends), the cells were switched to LK medium for 2 hr to down-regulate PAK-1 activity before treatment with HK or IGF-1. Cell viability was quantified by staining nuclei with 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) and counting of apoptotic nuclei as previously described (Koulich et al., 2001).

Western Blot Analysis

For whole-cell lysates, cells were washed once with ice-cold phosphate-buffered saline (PBS), followed by lysis with lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin (Cell Signaling), with a protease inhibitor cocktail tablet (Roche)]. The lysates were cleared by centrifugation for 10 min at 9,000*g* at 4°C. Sample buffer [3 × 187.5 mM Tris-HCl (pH 6.8) at 25°C, 6% sodium dodecyl sulfate (SDS), 30% glycerol, 150 mM dithiothreitol (DTT), 0.03% bromphenol blue] was added before heating to 95°C for 4 min. About 30 μg of whole-cell lysate was then separated by SDS-PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane (PVDF; Bio-Rad, Hercules, CA). After staining with Ponceau S to verify the uniformity of protein loads/transfer, the membranes were probed with the indicated antibody. All antibodies were used at 1:1,000 dilution, except for anti-PAK-1 (1:5,000) and anti-α-tubulin (1:15,000). Secondary antibodies were peroxidase-conjugated goat anti-rabbit IgG (sc-2004; 1:10,000), donkey anti-goat IgG (sc-2020; 1:10,000), and goat anti-mouse IgG (sc-2005; 1:10,000).

Transfection

Transient transfection of granule neurons was carried out as outlined by Koulich et al. (2001). Briefly, on day 5 after plating, 3.5 μg/well of DNA was precipitated by using the calcium phosphate method and added dropwise to cultures that had been incubated in BME for 30 min without serum or antibiotic. The precipitate was allowed to incubate with the cells for 30 min, and the cells were then washed three times with BME, and the original medium was replaced. On the next morning, cultures were switched to HK or LK and incubated for 24 hr, after which immunocytochemistry was performed. Immunocytochemistry and survival analysis were performed as describe by Koulich et al. (2001). The myc-tagged plasmids PAK-1T423E and PAK-1K299R were kind gifts of Dr. Gary M. Bokoch (Scripps Research Institute, La Jolla, CA).

Immunoprecipitation

Cells were treated and lysed as described above, up to the point of addition of sample buffer. One microgram of antibody was added to 300 μg of the cleared lysates and incubated on a rocker overnight at 4°C, followed by addition of 25 μl of protein A/G agarose beads (Santa Cruz Biotechnology) for 2 hr. The immune-bead complex was washed three times with lysis buffer, followed by the addition of sample buffer and SDS-PAGE.

In Vitro Kinase Assays

For kinase assays, after washes with lysis buffer, immunoprecipitates were washed three times with kinase buffer (50 mM HEPES, 10 mM MgCl, 10 mM β-glycerophosphate, 1 mM dithiothreitol). Washed immunoprecipitates were resuspended in 30 μl kinase buffer with 1 μM cold ATP, 1 μCi [γ-^{32}P]-ATP (MP Biomedicals, Irvine, CA), and 5 μg histone H4 (Roche) and incubated for 30 min at 30°C. The reaction was stopped by addition of sample buffer, followed by heating at 95°C for 4 min. The samples where then separated by SDS-PAGE and transferred electrophoretically to PVDF and developed by autoradiography. The same membranes were probed with PAK-1 antibody to demonstrate that similar amounts of PAK-1 were used for the assays.

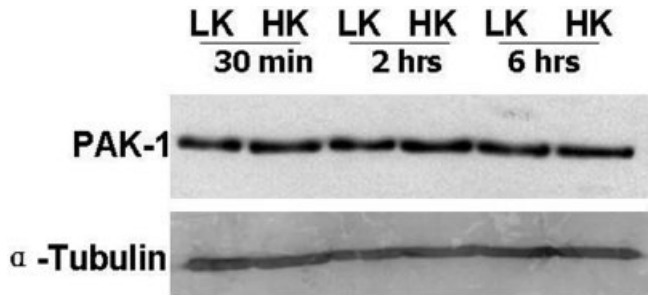

Fig. 1. Western blot analysis of PAK-1 expression. Cultures of cerebellar granule neurons were switched to LK or HK medium for the indicated periods. Whole-cell lysates were prepared and used for Western blot analysis with a PAK-1 antibody. The same blot was reprobed with an antibody against tubulin to show similar loading. PAK-1 expression was unchanged in neurons primed to die by LK treatment.

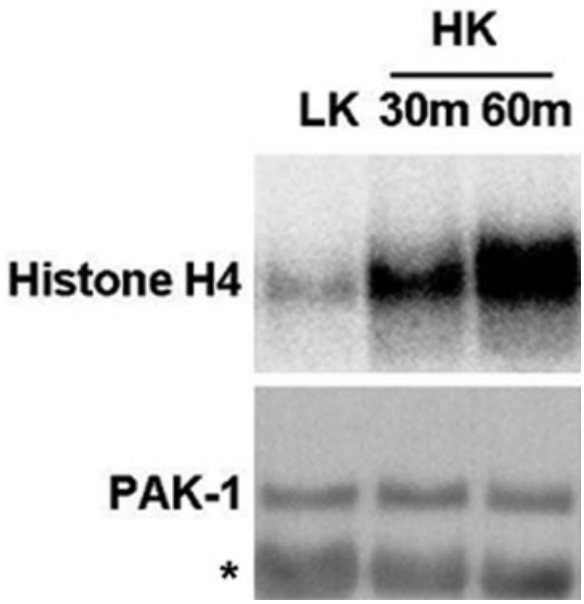

Fig. 2. Analysis of PAK-1 kinase activity following HK treatment. After 2 hr in LK medium, neuronal cultures were treated with HK or LK medium for 30 or 60 min. Whole-cell lysates were prepared and PAK-1 immunoprecipitated. The immunoprecipitated PAK-1 was used in an in vitro kinase reaction containing radiolabeled ATP and with histone H4 as substrate. After SDS-PAGE and transfer to PVDF membrane, the extent of histone H4 phosphorylation was visualized by autoradiography. The same membrane was probed with a PAK-1 antibody to demostrate equal pull-down. The dark band below the 68-kDa PAK-1 band corresponds to immunoreactivity to the heavy chain (marked with an asterisk). Treatment with HK stimulates PAK-1 activity.

RESULTS

Cerebellar granule neurons undergo apoptosis when switched from HK medium to medium containing LK (D'Mello et al., 1993). Although cell death begins at about 16 hr after LK treatment, commitment to death occurs by 6 hr (Borodezt and D'Mello,1998). We examined whether the level of PAK-1 expression was altered in neurons primed to die by LK treatment. As shown in Figure 1, PAK-1 levels were unchanged by LK treatment.

We next examined whether PAK-1 activity is regulated by HK. PAK-1 was immunoprecipitated from neuronal cultures that were treated with HK for 30 and 60 min and the activity of the enzyme examined in an in vitro kinase assay. As shown in Figure 2, PAK-1 activity was robustly activated by HK. Peak activity was found at 60 min after HK treatment (Fig. 2), and the activity remained elevated for at least 120 min (not shown).

The activation of PAK-1 by HK raised the possibility that PAK-1 activity was necessary for the survival-promoting effect of HK. To examine this issue, neuronal cultures were transfected with a plasmid overexpressing a dominant-negative form of PAK-1 (dn-PAK-1). As shown in Figure 3, overexpression of dn-PAK-1 substantially reduced the ability of HK to maintain neuronal survival. Furthermore, overexpression of a constitutively active form of PAK-1 (ca-PAK-1) was capable of maintaining survival even in LK medium. Taken together, these results show that PAK-1 is important for HK-mediated survival of cerebellar granule neurons.

PAK-1 serves as an effector for the Rho GTPases Rac1 and Cdc42, which have been reported to be necessary for the survival of cultured cerebellar granule neurons. Strong association between PAK-1 and Rac1 has previously been demonstrated (Manser et al., 1994). As a step toward investigating how PAK-1 was activated in neurons by HK, we examined whether it was associated with Rac1 and whether this association was perturbed by LK treatment. Although Rac1 expression is detectable in cerebellar granule neurons, an interaction between Rac1 and PAK-1 could not be detected. A similar analysis was performed for Cdc42 (Fig. 4A). Control experiments showed that PAK-1 did interact with Rac1 when overexpressed in the HEK293T cell line (Fig. 4B). As shown in Figure 4C, interaction between Cdc42 and PAK-1 was also undetectable in neuronal cultures (Fig. 4C). A control experiment similar to that shown in Figure 4 revealed interaction between PAK-1 and Cdc42 in HEK293T cells (not shown). GTPase-independent mechanisms of PAK-1 activation have recently been identified. One such mechanism involves interaction with the serine/threonine kinase PDK1 (King et al., 2000). As shown in Figure 4D, interaction between PDK1 and PAK-1 is detectable in neurons, although the level of interaction is not changed by LK treatment.

To gain insight into the mechanism by which PAK-1 mediates neuronal survival, we used pharmacological inhibitors of signaling proteins that are known to be important components of survival-promoting signaling pathways. The Raf-MEK-ERK pathway is one such pathway, and signaling by this pathway can be blocked by using PD98059, which is a highly selective inhibitor of MEK. We have previously found that, in cultured neurons, PD98059 efficiently inhibits ERK phosphorylation at a dose of 20 μM (Chin et al., 2004). As shown in

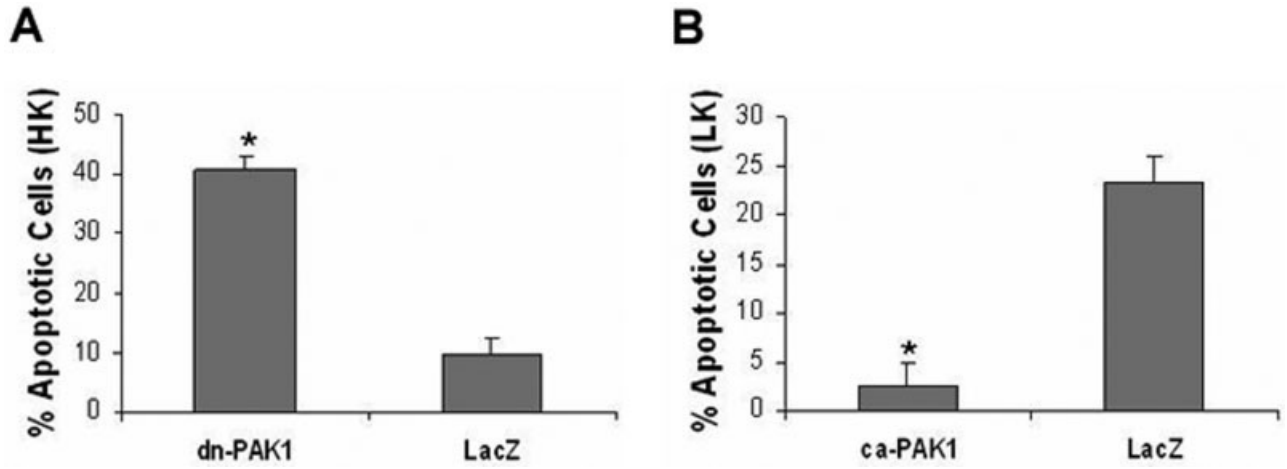

Fig. 3. Effect of dominant-negative PAK-1 and constitutively active-PAK-1 expression in neurons. Plasmid expression vectors encoding a myc-tagged dominant-negative form of PAK-1, pCMV-PAK-1K299R (**A**; dn-PAK-1), or a myc-tagged constitutively active form, pCMV-PAK-1T423E (**B**; ca-PAK-1), were used to transfect neuronal cultures. The cultures transfected with dn-PAK-1 and ca-PAK-1 were switched to HK or LK medium, respectively, for 24 hr. A lacZ-expressing vector, pCMV-LacZ, was used as a control. The proportion of transfected neurons (myc- or LacZ-positive) that were apoptotic (as judged by DAPI staining of nuclei) was quantified. $*P < 0.05$ vs. viability of LacZ-transfected cells using two-tailed Student's *t*-test, assuming unequal variance. Error bars are SEM of three independent experiments.

Figure 5, PD98059 had no effect on PAK-1-mediated neuronal survival when used at this dose. Another powerful antiapoptotic signaling pathway is the PI-3 kinase–Akt pathway. This pathway is necessary for the survival-promoting effect of IGF-1 in cultured cerebellar granule neurons (Datta et al., 1997; Dudek et al., 1997; D'Mello et al., 1997; Li et al., 2001; Chin and D'Mello 2004). Treatment with LY294002, a specific inhibitor of PI-3 kinase, which blocks IGF-1-mediated neuronal survival (D'Mello et al., 1997), failed to reduce the ability of PAK-1 to maintain neuronal survival (Fig. 5). In contrast, ML-9, a compound that has been used as an inhibitor of Akt (Hernandez et al., 2001; Smith et al., 2001; Desbois-Mouthon et al., 2002), inhibits survival promotion by PAK-1.

To examine whether PAK-1 was also involved in survival promotion by other survival factors, we investigated whether PAK-1 was also activated by IGF-1 treatment. As shown in Figure 6, IGF-1 treatment caused a robust activation of PAK-1. PAK-1 activation by IGF-1 peaked at 30 min and was down to control levels by 60 min. As observed with HK, interaction between PAK-1 and PDK1 was seen following IGF-1 treatment (Fig. 4A). Association with Rac1 and Cdc42 was, however, undetectable (Fig. 4C,D).

In contrast to work with PAK-1, which promotes cell survival, work by many investigators has shown that in cell lines PAK-2 can be cleaved during apoptosis by caspase-3 into a product lacking the regulatory domain and that is constitutively active (Lee et al., 1997; Rudel and Bockoch, 1997; Walter et al., 1998). In cerebellar granule neurons induced to undergo apoptosis by LK treatment, cleavage of PAK-2 is not detectable (Fig. 7), suggesting that caspase-mediated activation of PAK-2 does not contribute to the induction of apoptosis in neurons (Miller et al., 1997; Padmanabhan et al., 1999; D'Mello et al., 2000).

DISCUSSION

We show that PAK-1 is activated by both HK and IGF-1. Whereas IGF-1-mediated activation of PAK-1 is transient, peaking at about 30 min after treatment, stimulation by HK is more sustained. Overexpression of a dominant-negative form of PAK-1 blocks the survival-promoting effect of HK, suggesting an essential role for PAK-1 in depolarization-mediated cerebellar granule neuron survival. Overexpression of active PAK-1 can prevent apoptosis in the absence of any survival-promoting stimulus, implicating PAK-1 as a central molecule in neuronal survival.

Whether any of the other PAK members regulates neuronal survival is at present unclear. In nonneuronal cells, PAK-4 has also been found to be antiapoptotic. On the other hand, PAK-2 is proapoptotic. The proapoptotic function of PAK-2 is activated by caspase-3, which cleaves it into a 28-kDa N-terminus and a 34-kDa C-terminus fragment (Lee et al., 1997; Rudel and Bockoch, 1997; Walter et al., 1998). In cerebellar granule neurons, cleavage of the 62-kDa PAK-2 protein is not detectable following LK treatment. It deserves mention that, in contrast to other paradigms of neuronal apoptosis, such as nerve growth factor (NGF)-deprived sympathetic neurons, LK-induced apoptosis of cerebellar granule neurons is believed to be caspase independent (Miller et al., 1997; Padmanabhan et al., 1999; D'Mello et al., 2000).

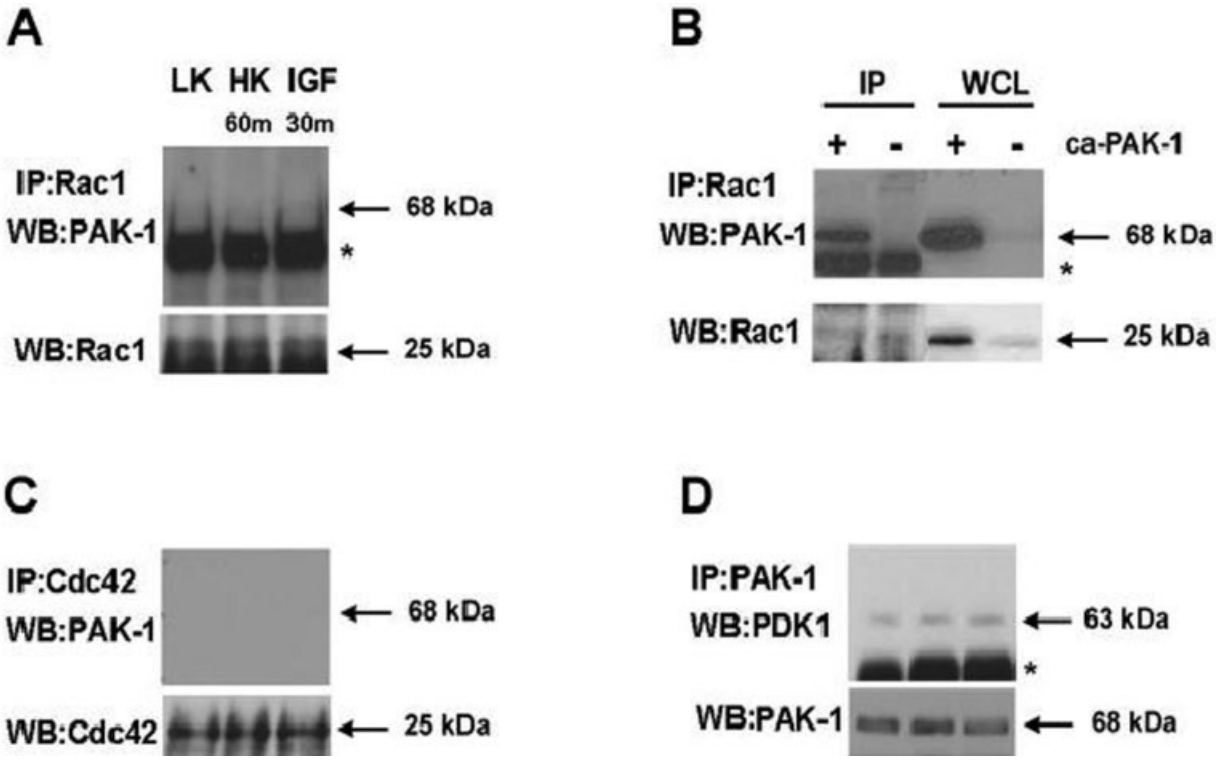

Fig. 4. Interaction between PAK-1 and Rac1, Cdc42, and PDK1. A,C,D: After LK treatment for 2 hr, cerebellar granule neuron cultures were treated with LK or HK medium for 60 min or with LK medium containing IGF-1 (50 ng/ml) for 30 min. Whole-cell lysates were prepared, and Rac1, Cdc42, and PAK-1 immunoprecipitated from 300 μg of the lysates. The immunoprecipitate was subjected to Western blot analysis and probed with antibodies against Rac1, Cdc42, PDK1, or PAK-1 antibody. The blot was reprobed with an appropriate antibody to show that similar amounts of protein were pulled down in the different lanes. In **A**, Rac1 was used to immunoprecipitate, and the Western blot was probed with PAK-1 and Rac-1. In **C**, Cdc42 was used to immunoprecipitate, and Western blot was performed with PAK-1 and Cdc42. In **D**, PAK-1 was used to immunoprecipitate, and Western blot was performed with PDK1 and PAK-1. The dark band of ~55 kDa (marked with an asterisk) in A, **B**, and D corresponds to heavy-chain immunoreactivity. D shows the results of a control experiment performed using the HEK293 cell line. Rac1 antibody was used to immunoprecipitate and then subjected to Western blot analysis with a PAK-1 antibody (under IP). Also loaded on the gel were preimmunoprecipitate aliquots of the whole-cell lysate (under WCL).

The signaling mechanism mediating the survival-promoting effect of PAK-1 warrants investigation. Previous studies have demonstrated the importance of the Raf-MEK-ERK signaling pathway in neuronal survival (Bonni et al., 1999). It is known that PAK-1 phosphorylates MEK1 at Ser298, leading to its activation (Frost et al., 1997). Direct phosphorylation and activation of Raf-1 by PAK-1 have also been demonstrated (Zang et al., 2002; Tran and Frost, 2003). It was thus possible that, in cerebellar granule neurons, PAK-1 promotes survival by activating the Raf-MEK-ERK pathway. Arguing against this possibility, however, is our finding that pharmacological inhibition of MEK-ERK signaling fails to reduce the survival by PAK-1.

Another well-established substrate of PAK-1 is the proapoptotic Bcl-2 protein Bad (Shurmann et al., 2000). PAK-1 phosphorylates Bad at Ser112 and Ser136, rendering it inactive. Previous studies conducted using cerebellar granule neurons have shown that Bad is phosphorylated by HK and IGF-1 at these residues (Datta et al., 1997; Gleichmann et al., 2000; Konishi et al., 2002). Finally, PAK-1 has been reported to be required for the activation of the nuclear factor-κB (NF-κB) transcription factor in nonneuronal cells (Frost et al., 2000). Constitutively active PAK-1 stimulates NF-κB activity (Foryst-Ludwig and Naumann, 2000; Frost et al., 2000). NF-κB is critical for the survival of cerebellar granule neurons (Koulich et al., 2001), raising the possibility that the survival-promoting effect of PAK-1 is mediated by NF-κB.

Although PAKs are usually considered to be downstream effectors of Rac and Cdc42, we were unable to detect association between PAK-1 and either Rac1 or Cdc42. It is possible that other Rac isoforms, such as Rac2 or Rac3, associate with PAK-1 in neurons. More recent research has identified a number of GTPase-independent activation mechanisms for PAKs. For example, PAK-1 has been shown to be a substrate for PDK1 (King et al., 2000). PDK1 also phosphorylates and activates Akt, a central component of the PI-3 kinase–Akt signaling pathway. We have detected interaction between PAK-1 and PDK1 in

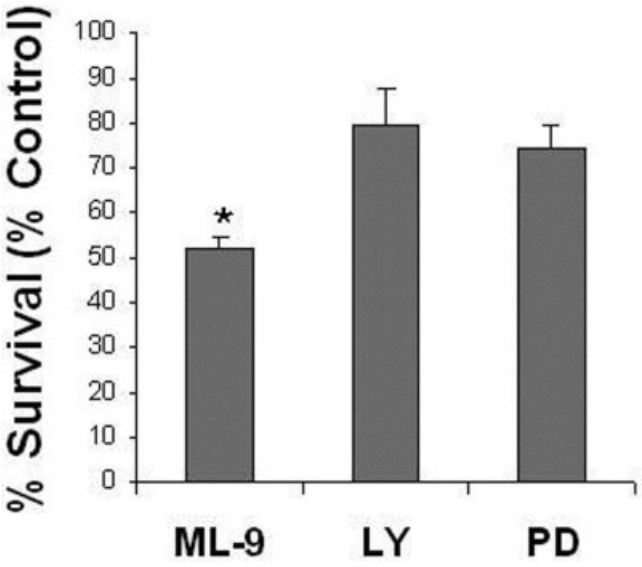

Fig. 5. Effect of PI-3 kinase, Akt, and MEK inhibition on PAK-1-mediated survival. Neuronal cultures were transfected with a myc-tagged and constiutively active form of PAK-1 (ca-PAK-1). The transfected cultures were then switched to LK medium containing 20 μM ML-9, 10 μM LY294002 (LY), or 20 μM PD98059 (PD) for 24 hr. Control cultures overexpressing ca-PAK-1 were treated with LK alone. After 24 hr, cells were fixed, and immunocytochemistry was performed. Transfected cells' viability was quantified by DAPI staining of nuclear DNA. $*P < 0.05$ vs. control cultures by Student's *t*-test. Error bars are SEM.

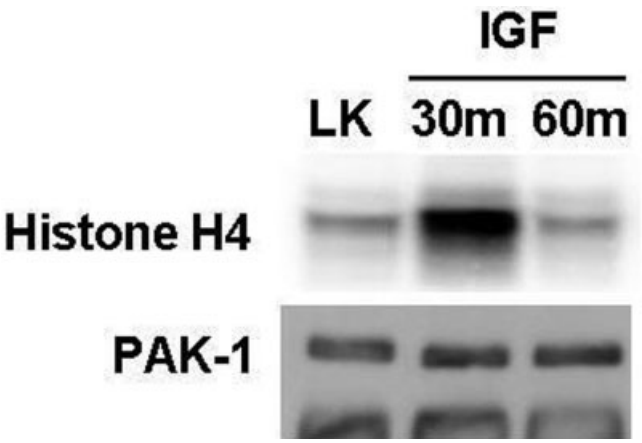

Fig. 6. IGF-1 stimulates PAK-1 kinase activity. Neuronal cultures were switched to LK medium for 2 hr, followed by treatment with 50 ng/ml of IGF-1 for 30 or 60 min. Whole-cell lysates were prepared and PAK-1 immunoprecipitated. The immunoprecipitated PAK-1 was used in an in vitro kinase reaction containing radiolabeled ATP and with histone H4 as substrate. After SDS-PAGE and transfer to PVDF membrane, the extent of histone H4 phosphorylation was visualized via autoradiography. The same membrane was probed with a PAK-1 antibody.

neurons. Previous studies have shown that the activation of PAK-1 by PDK1 is independent of PI-3 kinase and 3-phosphoinsotides (King et al., 2000). Consistent with this finding, we find that pharmacological inhibition of

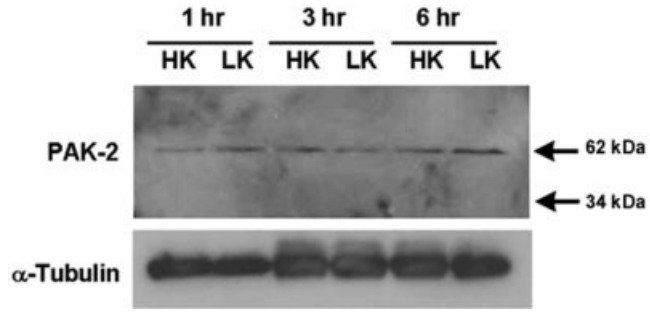

Fig. 7. Western blot analysis of PAK-2 following LK treatment. Cerebellar granule neurons were switched to LK medium for the periods shown. Whole-cell lysates were prepared and subjected to Western blot analysis using a PAK-2 antibody. No major change of expression or cleavage of the full form of PAK-2 was observed.

PI-3 kinase does not inhibit the ability of PAK-1 to promote neuronal survival. It is unclear whether the interaction with PDK1 is important for PAK-1-mediated neuronal survival. We have found that treatment with ML-9, which inhibits Akt activity in cerebellar granule neurons (Chin and D'Mello, 2004) and in other cell types (Hernandez et al., 2001; Smith et al., 2001; Desbois-Mouthon et al., 2002), blocks neuronal survival by PAK-1, suggesting the requirement for Akt.

In summary, we have identified PAK-1 as a molecule that is involved in the regulation of neuronal survival and one that is required for survival promotion by depolarization. Although further work will reveal the molecular mechanism by which PAK-1 acts to promote neuronal survival, our results suggest the involvement of PDK-1 and Akt.

ACKNOWLEDGMENTS

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Inhibition of neuronal apoptosis by the cyclin-dependent kinase inhibitor GW8510: Identification of 3′ substituted indolones as a scaffold for the development of neuroprotective drugs

Kyle Johnson, Li Liu, Nazanin Majdzadeh, Cindy Chavez, Paul C. Chin, Brad Morrison, Lulu Wang, Jane Park, Priti Chugh, Hsin-Mei Chen and Santosh R. D'Mello

Department of Molecular and Cell Biology, University of Texas at Dallas, Richardson, Texas, USA

Abstract

Increasing evidence suggests that neuronal apoptosis is triggered by the inappropriate activation of cyclin-dependent kinases leading to an abortive re-entry of neurons into the cell cycle. Pharmacological inhibitors of cell-cycle progression may therefore have value in the treatment of neurodegenerative diseases in humans. GW8510 is a 3′ substituted indolone that was developed recently as an inhibitor of cyclin-dependent kinase 2 (CDK2). We found that GW8510 inhibits the death of cerebellar granule neurons caused by switching them from high potassium (HK) medium to low potassium (LK) medium. Although GW8510 inhibits CDK2 and other CDKs when tested in *in vitro* biochemical assays, when used on cultured neurons it only inhibits CDK5, a cytoplasmic CDK that is not associated with cell-cycle progression. Treatment of cultured HEK293T cells with GW8510 does not inhibit cell-cycle progression, consistent with its inability to inhibit mitotic CDKs in intact cells. Neuroprotection by GW8510 is independent of Akt and MEK-ERK signaling. Furthermore, GW8510 does not block the LK-induced activation of Gsk3β and, while inhibiting c-*jun* phosphorylation, does not inhibit the increase in c-*jun* expression observed in apoptotic neurons. We also examined the effectiveness of other 3′ substituted indolone compounds to protect against neuronal apoptosis. We found that like GW8510, the VEGF Receptor 2 Kinase Inhibitors [3-(1H-pyrrol-2-ylmethylene)-1,3-dihydroindol-2-one], {(Z)-3-[2,4-Dimethyl-3-(ethoxycarbonyl)pyrrol-5-yl)methylidenyl]indol-2-one} and [(Z)-5-Bromo-3-(4,5,6,6-tetrahydro-1H-indol-2-ylmethylene)-1,3-dihydroindol-2-one], the Src family kinase inhibitor SU6656 and a commercially available inactive structural analog of an RNA-dependent protein kinase inhibitor 5-Chloro-3-(3,5-dichloro-4-hydroxybenzylidene)-1,3-dihydro-indol-2-one, are all neuroprotective when tested on LK-treated neurons. Along with our recent identification of the c-Raf inhibitor GW5074 (also a 3′ substituted indolone) as a neuroprotective compound, our findings identify the 3′ substituted indolone as a core structure for the designing of neuroprotective drugs that may be used to treat neurodegenerative diseases in humans.

Keywords: cerebellar granule neurons, cyclin-dependent kinases, neurodegeneration, neuronal apoptosis, neuroprotection, 3′ substituted indolones.

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A common feature of many neurological disorders is the decrease in nervous system functionality resulting from a loss of neurons. While drugs that provide symptomatic benefit for some neurodegenerative diseases are available, these do not slow down the pathological loss of neurons. A number of studies performed using animal models of neurodegenerative diseases have demonstrated that blocking neuronal death can improve behavioral outcome (reviewed in Saporito *et al*. 2002; Vila and Przedborski 2003). A substantial amount of pharmaceutical research on neurodegenerative diseases has therefore focused recently on identifying small molecule inhibitors of neuronal cell death.

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Address correspondence and reprint requests to Santosh R. D'Mello, Department of Molecular and Cell Biology, University of Texas at Dallas, 2601 N. Floyd Road, Richardson, TX 75083, USA.
E-mail: dmello@utdallas.edu

Abbreviations used: BME, basal minimal Eagle; CDK, cyclin-dependent kinases; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated protein kinase; FACS, fluorescence-activated cell sorter; HK, high potassium; JNK, Jun N-terminal kinase; LK, low potassium; MEK, mitogen-activated protein kinase; MLK, mixed lineage kinase; NCS, newborn calf serum; 3-NP, 3-nitropropionic acid; PBS, phosphate-buffered saline; PI-3, kinase, phosphatidylinositol 3-kinase.

Many of these studies have been performed using cell culture paradigms such as trophic factor-deprived sympathetic neurons, cerebellar granule neurons exposed to non-depolarzing medium and cortical neurons treated with DNA-depolarizing agents (reviewed in Deshmukh and Johnson 1997; D'Mello 1998; Mattson 2000; Chang *et al*. 2002).

A substantial body of evidence obtained largely from *in vitro* paradigms suggests that apoptosis-inducing stimuli might reactivate components of the cell-cycle machinery that are normally quiescent in mature neurons (reviewed in Liu and Greene 2001; O'Hare *et al*. 2002; Becker and Bonni 2004). Being post-mitotic, neurons are unable to completely traverse the cell cycle and instead, undergo apoptosis. Several studies have demonstrated the up-regulation of cyclins, cyclin-dependent kinases (CDKs), E2F or retinoblastoma protein (Rb) during neuronal apoptosis in culture systems (Freeman *et al*. 1994; Park *et al*. 1997a,b; Padmanabhan *et al*. 1999; O'Hare *et al*. 2000; Neystat *et al*. 2001; Konishi and Bonni 2003; Rideout *et al*. 2003). The induction and possible involvement of CDKs in neuronal loss has also been reported in *in vivo* models of degeneration (Cruz *et al*. 2003; Wen *et al*. 2004). Encouragingly, pharmacological inhibitors of CDKs protect against neuronal cell death caused by several different apoptotic stimuli (Park *et al*. 1997a,b; Padmanabhan *et al*. 1999; O'Hare *et al*. 2000; Neystat *et al*. 2001; Konishi and Bonni 2003; Rideout *et al*. 2003). The therapeutic value of such inhibitors for the treatment of neurodegenerative conditions is thus being actively considered (reviewed in O'Hare *et al*. 2002).

Among the most commonly used CDK inhibitors are flavoperidol (a flavonoid derivative; Losiewicz *et al*. 1994), roscovitine and olomoucine (both purine derivatives; Vesely *et al*. 1994; Meijer *et al*. 1997). Recently, a CDK inhibitor called GW8510 that is structurally different from the other known CDK inhibitors was identified (Davis *et al*. 2001). In this study, we examined whether GW8510 could protect neurons from apoptosis. We found that GW8510 blocks the death of cerebellar granule neurons induced to die by low potassium (LK) treatment. We also found, however, that although GW8510 inhibits several CDKs in kinase assays performed *in vitro*, treatment of neuronal cultures with this drug inhibits only CDK5 activity significantly. Because GW8510 is a 3′ substituted indolone that shares structural similarity with another neuroprotective drug, GW5074, that we recently identified (Chin *et al*. 2004), we examined whether other 3′ substituted indolone compounds were also neuroprotective. This analysis led to the identification of five other compounds capable of inhibiting neuronal apoptosis. More importantly, our analysis identified 3′ substituted indolones as a core structure on which neuroprotective drugs with potential therapeutic value could be designed.

Materials and methods

Materials

All tissue culture media and reagents were from Invitrogen (Carlsbad, CA, USA). GW8510 and GW5074 were from Sigma (St Louis, MO, USA), and all other indolone drugs used in this study were from Calbiochem (San Diego, CA, USA). The following antibodies were used: CDK1 (sc-954), CDK2 (sc-163), CDK4 (sc-601G) and CDK5 (sc-6247) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-Akt (#9471), p-ERK (#9101), p-MEK (#9121), p-GSK3 (#9331), p-c-Raf (#9421) and c-*jun* (#9162) (Cell Signaling, Beverly, MA, USA). All antibodies were used at 1 : 1000. Secondary antibodies (Santa Cruz) were peroxidase-conjugated goat anti-rabbit IgG (sc-2004; 1 : 10 000), donkey anti-goat IgG (sc-2020; 1 : 5000) and goat anti-mouse IgG (sc-2005; 1 : 10 000).

Neuronal cultures, treatments and cell viability assays

Cerebellar granule neurons were cultured from 7- to 8-day-old Wistar rats as previously described (D'Mello *et al*. 1993). The cells were plated in Basal Minimal Eagle (BME) medium, supplemented with 10% serum, 25 mM KCl, glutamine and gentamycin (D'Mello *et al*. 1993), in 24-well dishes (1×10^6 cells/well for viability assays), 60 mm dishes (10×10^6 cells/dish for western blots) and 100 mm dishes (25×10^6 cells/dish for immunoprecipitations). Cytosine arabinofuranoside (10 μM) was added to the culture medium 18–22 h after plating to prevent replication of non-neuronal cells. Treatments were performed 6–7 days after plating. For treatments, the cultures were switched to serum-free BME medium in the absence (LK medium) or presence of 25 mM KCl (HK medium). Candidate neuroprotective drugs were added to LK medium at the time the medium was switched. Cell viability was quantified using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide] assay or by staining nuclei with 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) as previously described (Koulich *et al*. 2001; Yalcin *et al*. 2003).

Adenoviral infection

The adenoviral expression vector encoding dominant-negative Akt was a gift from Wataru Ogawa (Kobe University, Hyogo, Japan). Infection of neurons with this vector was performed on day 5 after plating. The complete medium was set aside, then the neurons were washed twice with serum-free medium and 100 MOI of virus was added. After a 2 h incubation, the virus was washed off and the original medium was replaced. The protein was allowed to overexpress for 24 h before treatment was initiated.

Treatment of HEK293T cells

HEK293T cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 100 IU/mL penicillin, 100 μg/mL streptomycin and 10% newborn calf serum (NCS).

Treatment of HEK293T cells with GW8510 or roscovitine was performed using cells that were freshly split from subconfluent 100 mm dishes. The cells were pelleted by low speed centrifugation and washed twice with serum-free medium. Cells were then plated in 24-well dishes at a density of 1×10^5 cells/well. Serum (10% NCS), GW8510 and roscovitine, were added to the serum-free

medium at the time of plating. Viability was quantified by visualization of morphology. Similar results were obtained using the trypan blue exclusion assay.

Western blotting

Cells were treated as stated above and in figure legends, followed by two washes with cold phosphate-buffered saline (PBS); they were then lysed in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin; Cell Signaling) with a protease inhibitor cocktail tablet (Roche, Indianapolis, IN, USA). The lysates were centrifuged at 4°C at 9000 *g* for 10 min. Supernatant fluids were taken and protein amounts normalized by Bradford assay (500–0006; Bio-Rad, Hercules, CA, USA). For whole cell lysates, 30 μg were taken and 3× sodium dodecyl sulfate (SDS) sample buffer added (187.5 mm Tris-HCl pH 6.8 at 25°C, 6% SDS, 30% glycerol, 150 mM dithiothreitol (DTT), 0.03% bromphenol blue). The samples were then treated as described in Yalcin *et al*. (2003). Briefly, the lysates were heated at 95°C for 4 min, separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred electrophoretically to polyvinylidene difluoride membranes (PVDF; Bio-Rad). After staining with Ponceau S to check for uniformity of loading and transfer, membranes were incubated at 4°C with the indicated antibodies on a rocker overnight. The membranes were incubated with secondary antibody for 1 h at room temperature and developed by enhanced chemiluminesence (Amersham Bioscience, Piscataway, NJ, USA).

Activity of CDK and other kinases

For CDK activity assays, 300 μg total protein were taken and 1 μg of indicated antibody was added to the lysate, followed by agitation overnight at 4°C. The next morning, 25 μL protein A/G plus agarose beads (sc-2003, Santa Cruz) were added and the mixture rocked at 4°C for 2 h. The bead/immune complexes were pelleted and washed three times with the lysis buffer without a protease inhibitor cocktail tablet, followed by three washes with kinase buffer (40 mM Tris-HCl pH 7.5, 8 mM $MgCl_2$, 50 mM β-glycerol phosphate, 1 mM DTT). The immune complex was then resuspended in 30 μL kinase buffer plus 1 mM cold ATP, 1 μCi [γ-^{32}P] ATP and 5 μg histone H1 (Sigma), and incubated for 30 min at 30°C. The reaction was stopped by addition of 3× SDS–PAGE sample buffer (187.5 mM Tris-HCl, pH 6.8 at 25°C, 6% SDS, 30% glycerol, 150 mM DTT, 0.03% bromphenol blue). The samples were separated and transferred to PVDF membranes as described above. Following Ponceau S staining, membranes were visualized by autoradiography on a Storm 840 (Amersham Biosciences).

Other *in vitro* kinase assays were performed, with purified kinase and synthetic substrates under standard conditions, using the Kinase Profiling service of Upstate Biotechnology. For each assay, 5–10 mU purified kinase were used in a reaction mixture containing 100 μM ATP.

Fluorescence-activated cell sorter (FACS) analysis

HEK293T cells were treated with culture medium (DMEM with 10% NCS) plus 1 μM GW8510 or 50 μM roscovitine and harvested at a 24 h time point. Cells were washed twice with cold PBS and fixed in cold 70% ethanol at 4°C overnight. Approximately 10^6 cells were incubated with 100 μg/mL RNAse A (Roche) and 40 μg/mL propidium iodide (PI, Sigma); DNA content was analyzed by FACS using a FACS-Calibur instrument (BD Biosciences, San Diego, CA, USA). Data were plotted using CellQuest software (BD Biosciences); 10 000 events were analyzed for each sample. Data were also analyzed using the MODFit LT 3.0 software (BD Biosciences).

Results

GW8510 is protective against LK-induced apoptosis

Cultured cerebellar granule neurons undergo apoptosis when switched from HK medium to LK medium. As shown in

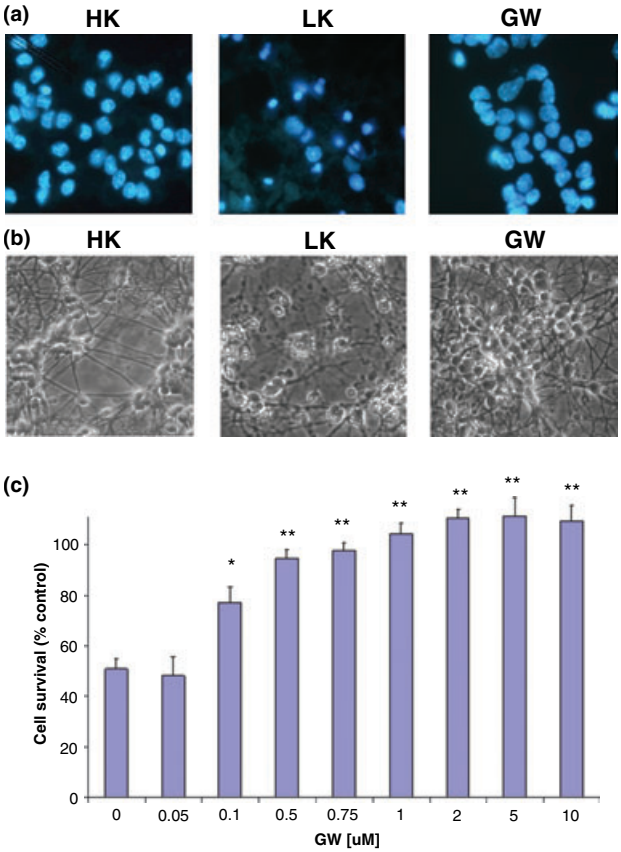

Fig. 1 GW8510 prevents LK-induced neuronal apoptosis. (a) DAPI staining of neuronal cultures treated with HK medium, LK medium, or LK medium containing 1 μM GW8510. Staining was performed 24 h after treatment. (b) Phase-contrast micrograph of cultures treated with HK medium, LK medium, or LK medium containing 1 μM GW8510 24 h after treatment. (c) Dose–response analysis showing effectiveness of various concentrations of GW8510. Viability was quantified 24 h after treatment and expressed as percentage of viability in HK. The results shown come from three independent experiments. $^*p < 0.05$ and $^{**}p < 0.005$ mean value ± standard deviation compared with viability of culture receiving LK.

Fig. 1, LK-induced apoptosis is inhibited by GW8510. Total protection was seen at doses of 1 μM and higher. The effect of GW8510 at higher doses was studied. No toxic effect was observed even when the drug was used at 10 μM. GW8510 had no effect on cultures treated with HK medium (data not shown).

GW8510 inhibits CDKs *in vitro* but has a weak effect on classical CDKs in cultured cells

GW8510 was discovered to be a potent inhibitor of CDK2 (IC50 = 60 nM) when tested *in vitro* (Davis *et al*. 2001). In addition to CDK2, Davis and co-workers reported that GW8510 also inhibited other CDKs, including CDKs 4 and 6, albeit at a higher concentration (Davis *et al*. 2001). To confirm and extend these results, we tested the effect of GW8510 on a variety of CDKs as well as on certain other kinases in assays utilizing purified kinases and synthetic peptide substrates. Consistent with the findings of Davis *et al*. (2001), GW8510 was most selective against CDK2. CDK1, CDK3 and CDK5 were also strongly inhibited by GW8510 when tested at 100 nM (Table 1), the dose at which a measurable protective effect of GW8510 on cultured neurons is observed. Besides CDKs 2, 3 and 5, moderate inhibition of CDK1 and Gsk3β was also observed (Table 1). A smaller but significant amount of inhibition was seen on c-Raf, JNK3, MKK7 and MSK1 (Table 1). In contrast, SAPK2a, SAPK2b, JNK1 and JNK2 were not inhibited by GW8510.

To confirm that GW8510 inhibited CDK2 and related CDKs, we used cell lysates prepared from cerebellar granule neurons treated with HK or LK medium. CDK2 and other CDKs were immunoprecipitated using specific antibodies and the effect of GW8510 on the immunoprecipitated kinase was studied. The activities of CDK2 and CDK5 were markedly increased in LK-treated cultures (Fig. 2). As observed with purified kinase, addition of GW8510 to CDK2 immunoprecipitated from LK-treated cultures inhibited its activity (Fig. 2a). Surprisingly, however, when GW8510 was added to cultures of neurons and the CDK2 immunoprecipitated from these drug-treated cultures, CDK2 activity was only modestly reduced. Similarly, CDK4 activity was inhibited when GW8510 was added directly to the immunoprecipitated kinase *in vitro*, but not inhibited

Table 1 All kinase assays were performed using 100 nM GW8510 and 100 μM ATP

Kinase	Activity (% control)
CDK1/cyclin B	33
CDK2/cyclin E	2
CDK3/cyclin E	5
CDK5/p35	8
c-Raf	68
Gsk3β	40
JNK1	93
JNK2	97
JNK3	74
MKK4	98
MKK6	110
MKK7	77
MSK1	78
SAPK2a	100
SAPK2b	98

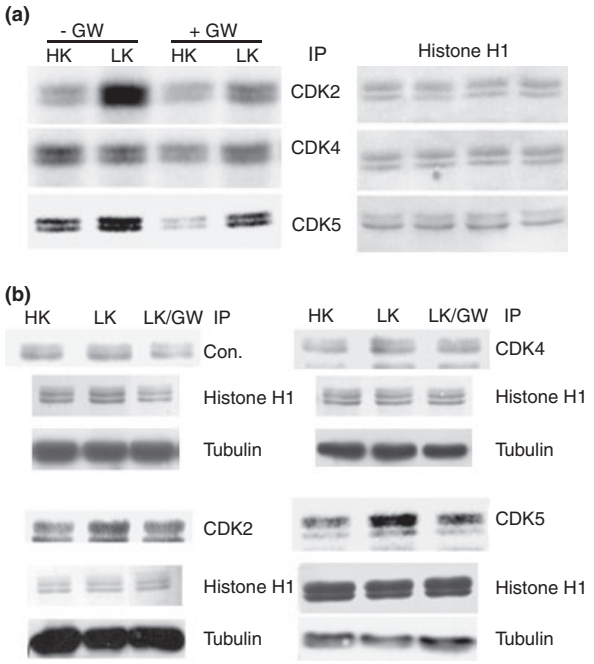

Fig. 2 Effect of GW8510 on activity of CDKs. (a) Effect of GW8510 on CDK2, 4 and 5 *in vitro*: Neuronal cultures were treated with HK or LK medium and whole cell lysates prepared. CDK2, CDK4 or CDK5 immunoprecipitated from the lysate, and aliquots of the kinases from HK and LK lysates, were used in *in vitro* kinase assays in the absence (– GW) or presence (+ GW) of 1 μM GW8510. Histone H1 was used as substrate. For CDK2 and CDK4, lysates from cultures treated for 6 h was used while for CDK5, lysates were prepared after 3 h of treatment. The panel showing Histone H1 is from the staining of the membrane with Coomassie blue and controls for the similarity in the amount of substrate used. (b) Activity of CDK2, 4 and 5 in GW8510-treated neuronal cultures. Neurons switched to HK, LK or LK medium containing 1 μM GW8510. CDK2, CDK4 and CDK5 were immunoprecipitated from whole cell lysates and used in kinase assays with Histone H1 as substrate. For CDK1 and CDK2, the cultures were treated for 6 h, while treatment for CDK5 assays was performed for 3 h. The panel showing Histone H1 is from staining of the membrane with Coomassie blue. A western blot of the supernatant fluid of the CDK immunoprecipitation was performed using a tubulin antibody to demonstrate that similar amounts of input lysate were used for immunoprecipitation.

when added to cultured neurons (Figs 2a and b). In contrast, CDK5, a non-mitotic and cytoplasmic CDK5 that is active only in neurons, is inhibited by GW8510 both *in vitro* and *in vivo* (Figs 2a and b). The inability to inhibit the activity of mitotic CDKs more strongly in intact neurons raised the possibility that neuroprotection by GW8510 was not due to a blockade of cell-cycle progression.

To investigate this possibility, we treated serum-stimulated HEK293T cells with GW8510 or roscovitine, a well established and commonly used CDK inhibitor that inhibits CDKs 1, 2 and 5 most potently (Meijer *et al*. 1997). Roscovitine is a purine derivative and thus, structurally distinct from GW8510. Inhibition of CDKs by roscovitine in HEK293T cells that are stimulated to proliferate leads to their death (Figs 3a and b). Treatment with GW8510, on the other hand, had no effect on cell viability (Figs 3a and b), consistent with the possibility that it does not inhibit cell-cycle progression efficiently. Even when used at 10 μM, a dose that is 10-fold higher than that at which it is neuroprotective, GW8510 did not affect the viability of HEK293T cells (Johnson and D'Mello, unpublished observation). To compare the effects of GW8510 and roscovitine more directly, we performed FACS analysis of HEK293T cells treated with the two drugs.

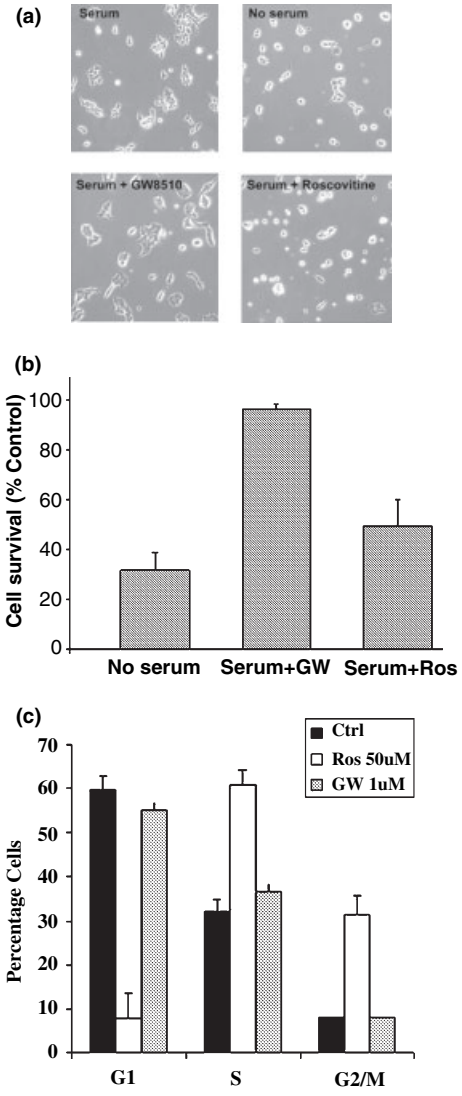

Fig. 3 Effect of GW8510 and roscovitine on HEK293T cell proliferation. (a) and (b) Freshly split HEK293T cells were plated in DMEM medium containing no serum or medium containing 10% serum, 10% serum plus 50 μM roscovitine or 1 μM GW8510. Viability was quantified 24 h later. (a) Phase-contrast appearance of cultures 24 h after treatment. (b) Viability of cultures expressed as percentage of control cultures which received medium containing 10% serum. Results come from three independent experiments performed in duplicate. (c) FACS analysis of cell-cycle progression. HEK293T cells were treated with 1 μM GW8510 (GW) or 50 μM roscovitine (Ros) or normal culture medium (DMEM with 10% NCS) for 24 h. Subsequently, cells were fixed and stained with propidium iodide and analyzed by FACS using MODfit LT 3.0. The values shown are averages (mean ± SD) of an experiment performed in triplicate. The data shown above are representative of three similar experiments.

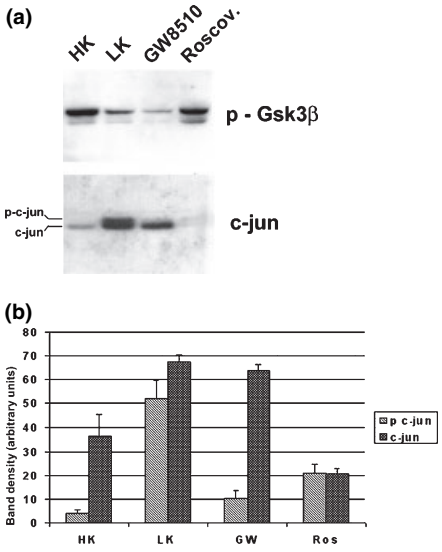

Fig. 4 GW8510 and roscovitine affect Gsk3β and c-*jun* differently. (a) Western blot analysis was performed with lysates obtained from neuronal cultures treated with HK medium (HK), LK medium (LK) or LK medium containing 1 μM GW8510 or 50 μM roscovitine (roscov) for 3 h. The figure shows the results obtained using antibodies against c-*jun* and phosphorylated Gsk3β. The dephosphorylation of Gsk3β by LK is potentiated by GW8510. In contrast, phosphorylation of Gsk3β remains elevated with roscovitine. With c-*jun*, GW8510 treatment inhibits LK-induced c-*jun* phosphorylation (upper band in doublet), but not the increase in expression (lower band). Roscovitine inhibits both phosphorylation and expression of c-*jun*. The experiment was repeated three times with similar results. (b) Quantification of c-*jun* phosphorylation induction. Density of the bands corresponding to c-*jun* and phospho-c-*jun* were quantified using Image Quant software. The graph shows results from three independent experiments. Standard bars represent SEM.

As shown in Fig. 3(c), treatment with roscovitine inhibits cell-cycle progression at the G1/S phase. In contrast, GW8510 treatment did not affect cell-cycle progression significantly, confirming that it did not inhibit mitotic CDKs in intact cells.

We compared the effects of GW8510 and roscovitine on specific signaling proteins known to regulate neuronal apoptosis. One of these molecules was Gsk3β, a protein implicated in the promotion of apoptosis in cerebellar granule neurons as well as other cell types. Activation of Gsk3β is inhibited by its phosphorylation at Ser9 (Li *et al*. 2000). Like HK, roscovitine treatment prevents the dephosphorylation of Gsk3β that occurs with LK treatment (Fig. 4). In contrast, dephosphorylation of Gsk3β is even more pronounced in cultures treated with GW8510.

C-*jun* is another molecule that is central to neuronal apoptosis (Estus *et al*. 1994; Ham *et al*. 1995; Watson *et al*. 1998). During apoptosis, c-*jun* is phosphorylated by jun N-terminal kinase (JNK; Eilers *et al.* 1998). Once activated by phosphorylation, c-*jun* can stimulate its own expression transcriptionally (reviewed in Barr and Bogoyevitch 2001). As described by other investigators (Ham *et al*. 1995; Watson *et al*. 1998), LK treatment of cerebellar granule neurons leads to an increase in both phosphorylation and its expression. Both of these changes are inhibited by HK and roscovitine (Figs 4a and b). While clearly preventing c-*jun* phosphorylation, treatment with GW8510 does not inhibit the increase in c-*jun* expression (Figs 4a and b). Although GW8510 and roscovitine affect Gsk3β and c-*jun* differently, they did have similar effects on certain other molecules (see below).

GW8510 acts in a MEK-ERK and Akt-independent manner

We examined whether treatment of neurons with GW8510 had any stimulatory effect on components of anti-apoptotic signaling pathways. The Raf-MEK-ERK pathway is known to mediate neuroprotection in some systems (Bonni *et al*. 1995). Activation of c-Raf depends on the dephosphorylation of the protein at an inhibitory site, Ser259 (reviewed in Dhillon and Kolch 2002; Hindley and Kolch 2002). As shown in Fig. 5, switching of cerebellar granule neurons to LK medium results in higher phosphorylation of Ser259, indicating lower activity of c-Raf in this condition. Not surprisingly, the level of MEK and ERK phosphorylation (which is necessary for the activation of these kinases) is also lower in LK (Fig. 5). GW8510 treatment fails to prevent the increase in c-Raf phosphorylation at Ser259, or the decline in MEK and ERK phosphorylation that occurs following LK treatment (Fig. 5).

To confirm that Raf-MEK-ERK signaling was not required for neuroprotection by GW8510, we used U0126, a potent and specific chemical inhibitor of MEK. U0126 did not affect neuroprotection by GW8510 (Fig. 6). As observed in other cell types, we have confirmed that U0126 completely blocks

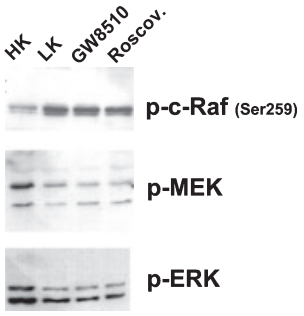

Fig. 5 GW8510 does not activate Raf-MEK-ERK signaling. Neuronal cultures were switched to HK medium (HK), LK medium (LK) or LK medium containing 1 μM GW8510 or 50 μM roscovitine (roscov). Whole cell lysates were prepared 3 h after treatment and analyzed by western blot analysis using antibodies specific for c-Raf phosphorylated at Ser259, phosphorylated MEK and phosphorylated ERK. The reduction in phosphorylation of MEK and ERK, and increased c-Raf^{Ser259} phosphorylation occurring after LK treatment, is not affected by GW8510.

MEK activity when used at 10 μM (Chin *et al.* 2004). Similar results were also observed with PD98059, another MEK inhibitor that is structurally distinct from U0126 (Fig. 6). Thus, the Raf-MEK-ERK pathway is not necessary for GW8510-mediated neuroprotection.

Another powerful anti-apoptotic signaling pathway is the PI-3 kinase–Akt pathway. In cultured cerebellar granule neurons, this pathway is necessary for the survival-promoting effect of IGF-1, but not of HK or cyclic AMP (D'Mello *et al*. 1997; Datta *et al*. 1997; Dudek *et al*. 1997; Liu and Greene 2001; Chin *et al*. 2004). In contrast to IGF-1, which stimulates Akt phosphorylation and activity, switching of cerebellar granule neurons from the medium containing serum and elevated potassium in which they are plated to serum-free HK or LK medium leads to a reduction in Akt phosphorylation at Ser473 (Kumari *et al*. 2001; Fig. 7). Treatment with GW8510 failed to prevent the reduction in Ser473 phosphorylation, although in comparison with HK or roscovitine, the decline in phosphorylation was delayed (compare phosphorylation at 1 h vs. 3 h in Fig. 7). To

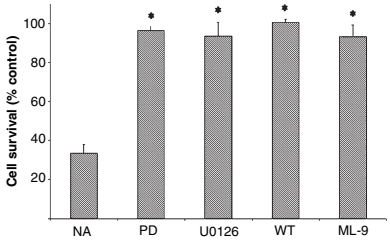

Fig. 6 Neuroprotection by GW8510 is not inhibited by pharmacological inhibition of MEK and PI-3 kinase–Akt signaling. Neuronal cultures were switched to LK medium containing no additives or supplemented with 1 μM GW8510 and 40 μM PD98059 (PD), 10 μM U0126, 100 nM Wortmannin or 20 μM ML-9. Cell viability was quantified 24 h later. Control cells received LK medium plus 1 μM GW8510.

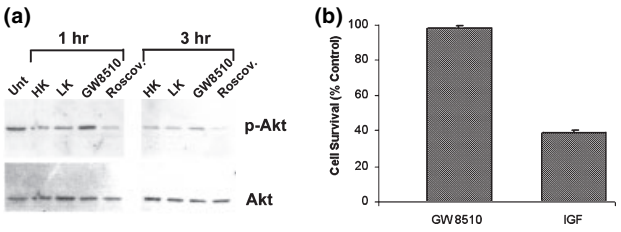

Fig. 7 Neuroprotection by GW8510 is Akt-independent. (a) Western blot analysis of Akt phosphorylation. Seven-day-old neurons plated in medium containing 10% FCS and 25 mM KCl cultures were either untreated (Unt) or switched to HK medium (HK), LK medium (LK), or LK medium containing 1 μM GW8510 or 50 μM roscovitine (roscov). Whole cell lysates were prepared 1 h and 3 h after treatment and the phosphorylation of Akt analyzed by western blotting using an antibody specific for phospho-Akt$^{Ser\ 473}$. The same blot was reprobed with total Akt. Although GW8510 can prevent Akt dephosphorylation at 1 h, by 3 h this effect is lost. (b) Neuronal cultures were infected with an adenoviral construct expressing a dominant-negative Akt (dn-Akt) construct that was HA-tagged. The cultures were then switched to LK medium containing 1 μM GW8510 or 50 ng/mL IGF-1. After 24 h, the proportion of infected cells (as judged by positive HA immunoreactivity) that was apoptotic (as judged by nuclear condensation or fragmentation visualized by DAPI-staining) was quantified. While dn-Akt inhibited IGF-1-mediated neuronal survival, it had no effect on survival by GW8510.

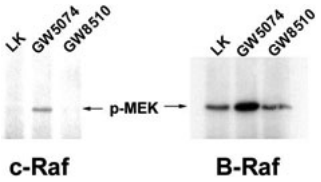

Fig. 8 Effect of GW5074 and GW8510 on B-Raf and c-Raf activity. Neurons were either untreated or switched to LK medium containing no additives (LK) or LK containing 1 μM GW5074 or 1 μM GW8510. After 1 h of treatment, the cultures were lysed and B-Raf or c-Raf immunoprecipitated from the lysate. The immunoprecipitated kinase was washed repeatedly and used in an *in vitro* kinase assay with MEK as substrate. The reaction mixture was subjected to western analysis using a phospho-MEK antibody as probe. The stimulation of c-Raf by GW5074 is caused by a build-up of activating modifications within neurons in response to treatment with the inhibitor, and is revealed by the washing off of the drug during the process of immunoprecipitation (see Hall-Jackson 1999a,b; Chin *et al.* 2004).

examine further whether PI-3 kinase–Akt signaling is involved in GW8510-mediated neuroprotection, we used pharmacological inhibitors of these kinases. Wortmannin, a highly selective inhibitor of PI-3 kinase, blocks IGF-1-mediated survival of granule neurons (D'Mello *et al.* 1997). As shown in Fig. 6, neuroprotection by GW8510 is not affected by wortmannin treatment. Similarly, ML-9, a compound that has been used as an inhibitor of Akt (Hernandez *et al.* 2001; Desbois-Mouthon *et al.* 2002; Smith *et al.* 2000), has no effect on GW8510-mediated survival (Fig. 6). To rule out PI-3 kinase–Akt signaling as being responsible for neuroprotection by GW8510, we used a dominant-negative (dn) form of Akt. Overexpression of dn-Akt did not interfere with the ability of GW8510 to prevent LK-induced neuronal death, although it did block IGF-1 mediated survival (Fig. 7). Thus, the PI-3 kinase–Akt signaling pathway is not required for GW8510-mediated neuroprotection.

Comparison of GW8510 with GW5074

We recently reported that another compound, GW5074, has strong neuroprotective effects in tissue culture paradigms of neuronal apoptosis (Chin *et al.* 2004). GW5074 also prevents neurodegeneration and improves behavioral outcome in an *in vivo* experimental model of Huntington's disease (Chin *et al.* 2004). GW5074 is a specific inhibitor of c-Raf, with no effect on the activity of CDKs or several other protein kinases that were tested (Lackey *et al.* 2000; Chin *et al.* 2004). While inhibiting c-Raf activity *in vitro*, treatment of neurons with GW5074 caused a paradoxical activation of c-Raf when measured *in vitro* in the absence of the drug (Chin *et al.* 2004 and Fig. 6). Such a paradoxical effect on c-Raf activity is also seen with other pharmacological inhibitors of c-Raf (Hall-Jackson *et al.* 1999a,b). As shown in Fig. 8, the paradoxical activation of c-Raf by GW5074 is not seen with GW8510 treatment. As previously reported, GW5074 treatment causes an activation of B-Raf (which is not inhibited by this drug), explaining the activation of MEK and ERK by this drug (Chin *et al.* 2004). As shown in Fig. 8, GW8510 has no effect on B-Raf activity. Hence, GW8510 acts by a mechanism that is distinct from that activated by GW5074.

The indolone moiety is associated with neuroprotection

GW8510 and GW5074 are both 3′ substituted indolone derivatives. Indolone derivatives are known to have potent and selective inhibitory activity towards different receptor tyrosine kinases (Mohammadi *et al.* 1997; Sun *et al.* 1998, 2000). The selectivity of these compounds towards specific kinases depends on the substituents on the indolone core, especially at the C-3 position (Mohammadi *et al.* 1997; Sun *et al.* 1998, 2000). Since the structural commonality between GW8510 and GW5074 is the indolone moiety, we investigated the possibility that the indolone moiety might serve as a core chemical structure for neuroprotection. We therefore tested the efficacy of several other commercially available 3′ substituted indolone derivatives against LK-induced neuronal death (see structures in Fig. 9a). One of these was oxindole-1 [3-(1H-pyrrol-2-ylmethylene)-1,3-dihydroindol-2-one], a potent and selective inhibitor of the VEGF receptor-2 tyrosine kinase (VEGF-R2; Sun *et al.* 1998; Kent *et al.* 1999). As shown in Fig. 9(b), LK-induced death of granule neurons was inhibited by treatment with oxindole-1. Maximum protection was seen at a dose of 10 μM. As seen with

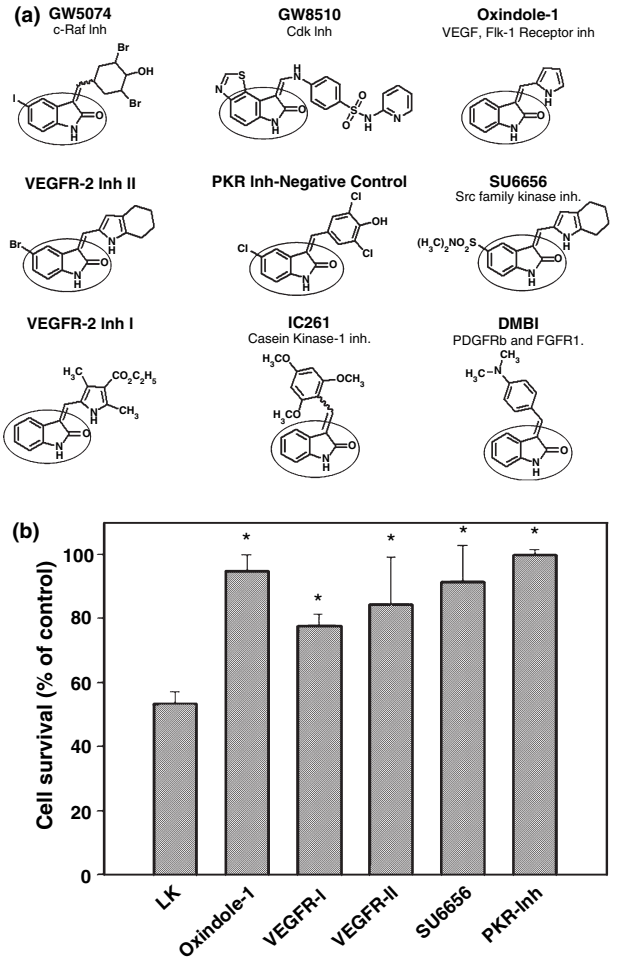

Fig. 9 Structures and neuroprotective efficacy of indolone compounds.Structures of indolone compounds described in this study. Neuroprotection by GW5074 was reported recently (Chin *et al.* 2004). The indolone moiety common to all the compounds is circled. **(b)** Effect of various indolones on LK-induced neuronal death. Neurons were switched to LK medium or LK medium containing 10 μM oxindole-1, 2.5 μM VEGF receptor 2 kinase inhibitor-I, 20 μM VEGF receptor 2 kinase inhibitor-II, 5 μM SU6656 and 2.5 μM PKR inhibitor-negative control. Control cells received HK medium. The doses used were selected based on dose–reponse analysis and found to be the most efficacious for the specific drug. The results shown are from four independent experiments. $^*p < 0.05$ ± SD compared with viability of culture receiving LK.

GW8510 and GW5074, treatment with oxindole-1 does not activate Akt or MEK-ERK signaling (data not shown).

As shown in Fig. 9(b), almost complete protection was also seen with VEGF receptor 2 kinase inhibitor-II [(Z)-5-Bromo-3-(4,5,6,6-tetrahydro-1H-indol-2-ylmethylene)-1,3-dihydroindol-2-one], a 3′ substituted indolone which also inhibits VEGF-R2, as well as other split domain receptor tyrosine kinases including FGF-R1 and PDGF-Rβ (Sun *et al.* 2000). In comparison, another selective inhibitor of VEGF-R2, {(Z)-3-[2,4-Dimethyl-3-(ethoxycarbonyl)pyrrol-5-yl)methylidenyl]indol-2-one}, designated as VEGF receptor kinase inhibitor-I (Sun *et al.* 1998), had more modest but significant neuroprotective activity. While VEGF receptor 2 kinase inhibitor-I was most effective at 2.5 μM (with toxicity at doses = 10 μM), VEGF receptor 2 kinase inhibitor-II displayed maximum protection at 20 μM (with no protection at doses < 10 μM). Previous work has shown that both drugs inhibit VEGF-R2 with similar potency (IC50 = 70 nM; Sun *et al.* 1998, 2000).

Table 2 All kinase assays were performed using 100 μMATP. VEGF receptor 2 kinase Inhibitor I (VEGFR2-InhI), VEGF receptor 2 kinase inhibitor II (VEGFR2-InhII) and PKR inhibitor-negative control were used at 0.5 μM,4 μM and 0.5 μM, respectively

	Activity (% control)		
Kinase	VEGFR2-InhI	VEGFR2-InhII	PKR-neg
CDK1/cyclin B	110	111	116
CDK2/cyclin A	101	84	96
CDK5/p35	92	115	111

Robust neuroprotection was also observed with SU6656, a potent Src family kinase inhibitor, and with 5-Chloro-3-(3,5-dichloro-4-hydroxybenzylidene)-1,3-dihydro-indol-2-one, a commercially available compound sold as an inactive structural analog of an RNA-dependent protein kinase (PKR) inhibitor (Fig. 9b). Two other indolone compounds that were tested, IC261, a casein kinase-1 inhibitor and DMBI, an inhibitor most selective against PDGF-R and FGF-R1, did not show appreciable protection (data not shown).

Since GW8510 inhibited CDK activity at least *in vitro*, we investigated whether some of the other neuroprotective compounds also inhibited CDKs, thus providing a possible explanation for how they acted to prevent apoptosis. Three of the other neuroprotective compounds, VEGF receptor 2 kinase (VEGF-R2) inhibitor I, VEGF-R2 inhibitor II and inactive PKR inhibitor, were chosen and their effects tested on the activity of three CDKs that are inhibited by GW8510 *in vitro*. As shown in Table 2, none of the three compounds had a signifi cant effect on the activities of CDK1, CDK2 or CDK5 when assayed *in vitro*. A similar lack of inhibition by these three compounds was observed in kinase assays using CDK2 and CDK5 that were immunoprecipitated from granule neurons (data not shown).

Discussion

We report here the identification of a small molecule chemical compound, GW8510, that prevents LK-induced death of cerebellar granule neurons. GW8510 was discovered as an inhibitor of CDK2 by Davis *et al.* (2001). The drug was also reported to affect other CDKs and to prevent chemotherapy-induced alopecia in rats, although this biological activity of GW8510 could not be reproduced and the

original report describing this drug was subsequently retracted in its entirety (Davis *et al*. 2002).

Results from several studies have suggested that the activation of mitotic CDKs in post-mitotic neurons can trigger apoptosis. Not surprisingly, treatment with pharmacological inhibitors of CDKs are protective in experimental paradigms of neurodegeneration. Although GW8510 strongly inhibits mitotic CDKs when added to immunoprecipitated or purified kinase *in vitro*, it does not have a significant inhibitory effect on the activity of CDKs when added to neuronal cultures. In contrast to mitotic CDKs, treatment of neuronal cultures with GW8510 does inhibit CDK5, a non-classical CDK whose activity is restricted to the nervous system. Although we do not have an explanation for this, it is possible that GW8510 does not enter the nucleus, or is actively extruded from it. In contrast to classical CDKs that are nuclear, CDK5 is localized primarily in the cytoplasm.

We compared the effect of GW8510 with that of roscovitine, a well studied and widely used CDK inhibitor. While roscovitine treatment kills HEK293T cells that are stimulated to proliferate with serum, treatment with GW8510 does not. FACS analysis confirmed that GW8510 does not block cell-cycle progression while treatment with roscovitine does. This, along with our findings that roscovitine and GW8510 have different effects on specific signaling molecules such as Gsk3β and c-*jun*, and that mitotic CDKs are not inhibited by GW8510 in neurons, strengthens the possibility that neuroprotection by GW8510 may occur by a mechanism not involving cell-cycle inhibition. It is possible that the neuroprotective effect of GW8510 is due to CDK5 inhibition. Increased CDK5 activity has been observed in a number of experimental paradigms of neurodegeneration and in brain tissue of patients with Amyotrophic Lateral Sclerosis and Alzheimer's disease (Patrick *et al*. 1999; Patzke and Tsai 2002; reviewed in Weishaupt *et al*. 2003). Preliminary evidence we have obtained suggests that GW8510 inhibits mixed-linage kinase-3 (MLK-3) when tested *in vitro* (Morrison and D'Mello, unpublished observation). MLK-3 has been shown to have pro-apoptotic effects in neurons, and pharmacological inhibition of MLKs is protective in a variety of *in vitro* and *in vivo* paradigms of neurodegeneration (Glicksman *et al*. 1998; Maroney *et al*. 1998; Xu *et al*. 2001; Harris *et al*. 2002). Whether endogenous MLK-3 activity or the activity of other MLKs is inhibited by GW8510 has not been tested.

We recently showed that a highly specific c-Raf inhibitor, GW5074, has strong neuroprotective effects in culture and in an animal model of neurodegeneration (Chin *et al*. 2004). Although GW8510 does not inhibit c-Raf in cultured neurons and GW5074 has no effect on CDKs (including CDK5), these drugs share structural similarity. Both compounds are 3′ substituted indolone derivatives. We tested whether other commercially available indolone compounds also possessed neuroprotective properties. We found that in addition to GW5074 and GW8510, five other 3′ substituted indolone derivatives, oxindole-1, VEGF receptor kinase inhibitor-I, VEGF receptor kinase inhibitor-II, PKR-1 inhibitor-negative control and SU6656, all protected cerebellar granule neurons against LK-induced apoptosis. Since the first three of these compounds inhibit VEGF-R2, it is possible that neuroprotection by these and by some of the other indolone compounds is due to the inhibition of VEGF signaling. Arguing against this possibility is our finding that treatment of cerebellar granule neuron cultures with VEGF either in LK or in HK has no effect on cell viability (unpublished observation). Moreover, while two of the VEGF-R2 inhibitors inhibit VEGF-R2 with the same potency, there is an approximately 10-fold difference in the concentration at which they are maximally neuroprotective. Another possibility to explain how these different inhibitors all exert neuroprotective effects is that although more selective for other kinases, some of the neuroprotective compounds identified in this study might also inhibit CDKs. We have previously reported that GW5074 has no inhibitory effect on CDK activity (Chin *et al*. 2004). The testing of VEGF-R2 inhibitor-I, VEGF-R2 inhibitor-II and PKR-1 inhibitor-negative control also showed no CDK inhibition, discounting the possibility that an ability to inhibit CDKs might represent a common property of these compounds.

Two other indolone compounds, IC261 and DMBI, were found to have no protective effect on LK-treated cerebellar granule cultures. Taken together, these findings suggest that the indolone moiety contributes to, but is not sufficient for, neuroprotection. Biochemical as well as X-ray crystallographic studies using different indolone compounds have shown that the indolone core associates with the ATP-binding site of various protein kinases, and that these compounds inhibit the enzyme by competing with ATP (Mohammadi *et al*. 1997; Sun *et al*. 1998, 2000; Davis *et al*. 2001). While our studies do not reveal the mechanism by which indolone compounds may confer neuroprotection, the finding that at least two of the compounds that we tested failed to prevent cell death suggests that protection is not due to a non-selective inhibition of protein kinases. This conclusion is strengthened by the finding that at high concentration, at which greater non-selectivity in their inhibitory action could be expected, some of these compounds are toxic (data not shown). Finally, based on an analysis of over 15 different kinases, at least one of the neuroprotective indolones (GW5074) is specific against c-Raf (Lackey *et al*. 2000; Chin *et al*. 2004).

Neurodegenerative diseases disrupt the quality of the lives of patients and cost society billions of dollars annually. Drugs that inhibit neuronal apoptosis could be candidates for therapeutic intervention in these disorders, and several small molecule compounds have recently been identified that protect against neuronal apoptosis in cell culture paradigms.

In this report we identify six additional neuroprotective compounds. All six of these compounds, along with GW5074, a drug that was recently demonstrated to be neuroprotective (Chin *et al*. 2004), are 3′ substituted indolones. Our findings therefore identify 3′ substituted indolones as a versatile scaffold for the development of additional and more effective neuroprotective compounds that could have therapeutic value in the treatment of neurodegenerative conditions.

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Research report

Inhibition of GSK3β is a common event in neuroprotection by different survival factors

Paul C. Chin[1], Nazanin Majdzadeh[1], Santosh R. D'Mello*

Department of Molecular and Cell Biology, University of Texas at Dallas, 2601 N. Floyd Road, Richardson, TX 75083, USA

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Abstract

Depolarizing concentrations of potassium (HK, 25 mM), cyclic AMP elevating agents and analogs (cAMP), insulin-like growth factor-1 (IGF-1), or lithium can maintain the survival of cultured rat cerebellar granule neurons (CGNs). We investigated the possibility that the signal transduction pathways utilized by these four survival factors converge in regulating a common molecular target. We targeted the regulation of the kinase GSK3β as the critical event in the survival directed by the four survival factors. We found that treatment of CGNs with HK, the cAMP-elevating agent forskolin, IGF-1, and lithium resulted in phosphorylation of GSK3β at serine-9 and thus its inactivation. Furthermore, pharmacological inhibition of core components in the survival signaling cascades initiated by HK, forskolin, IGF-1, and lithium causes apoptosis and activation of GSK3β accompanies this death. Finally, we examined the pharmacological inhibitors of GSK3β, GSK3 inhibitor I, TDZD-8, and SB-415286, for their ability to prevent low potassium (LK)-induced apoptosis. Although previous reports demonstrate inhibition of GSK3β in in vitro kinase assays with GSK3 inhibitor I and TDZD-8, we were unable to detect inhibition of GSK3β in neuronal cultures treated with these compounds and thus no protection from LK-induced apoptosis. SB-415286 on the other hand, was able to rescue CGNs from cell death. Taken together, we conclude that regulation of GSK3β is a critical convergence event in the promotion of CGN survival by different factors.

Theme: Development and regeneration
Topic: Neuronal death

Keywords: GSK3β; HK; IGF-1; cAMP; Lithium; Cerebellar granule neuron; Apoptosis

1. Introduction

Glycogen synthase kinase 3 (GSK3) was originally identified for the role it played in the regulation of glycogen metabolism (reviewed in [11,14,15]). Gradually, the versatility of this molecule became apparent as its role in other cellular processes was identified. GSK3 has been shown to participate in the regulation of protein synthesis, cell proliferation, cell differentiation, microtubule dynamics, cell motility, and apoptosis (reviewed in [11,14,15]). There are two known isoforms of GSK3 in mammals encoded by distinct genes, GSK3α and GSK3β, with molecular weights of 51 and 47 kDa, respectively [34]. The two proteins are structurally similar, yet they are functionally different. Although a role for GSK3α in the generation of amyloid-β peptides has been suggested [31], the functional significance of GSK3α in the regulation of apoptosis is unclear at best. However, disruption of the GSK3β gene in mice results in embryonic lethality associated with massive apoptosis in the liver [17]. Mice deficient in GSK3β are unable to elicit an anti-apoptotic response via nuclear factor κB (NF-κB) to tumor necrosis factor α (TNFα), indicating a pro-survival role for GSK3β in the developing embryo [17]. In stark contrast to the pro-survival role of GSK3β in liver

Abbreviations: cAMP, cyclic adenosine monophosphate; GSK3, glycogen synthase kinase 3; NF-κB, nuclear factor-κB; PI-3K, phosphatidylinositide-3 kinase

* Corresponding author. Fax: +1 972 883 2409.
E-mail address: dmello@utdallas.edu (S.R. D'Mello).
[1] These authors contributed equally to the work.

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development, a substantial body of evidence indicates that activation of GSK3β promotes apoptosis in neurons and other cell types. Thus, the role of GSK3β in apoptosis is important but tissue specific.

In the context of neuronal apoptosis, the phosphatidylinositide-3 kinase (PI3-K) pathway is the best characterized survival signaling cascade regulating the activity of GSK3. Activated by growth factors, such as IGF-1 or insulin, stimulation of Akt by this cascade results in inactivation of GSK by phosphorylation of N-terminal serine residues (serine-21 on GSK3α and serine-9 on GSK3β) [6]. In addition, inactivation of GSK3β by protein kinase A (PKA) results in neuronal survival in response to elevated levels of cAMP [24]. Lithium is a powerful mood stabilizer and inhibits GSK3β directly and indirectly (reviewed in [18,25]). Lithium competes with magnesium (Mg^{++}) for binding to GSK3β leading to inhibition, and lithium treatment leads to phosphorylation/inactivation of GSK3β in CGNs by a mechanism involving Akt ([29]; reviewed in [11]). These observations suggest that GSK3β plays a critical role in the promotion of apoptosis in neurons and suppression of apoptosis signaling must involve inhibition of GSK3β.

When cultures of rat cerebellar granule neurons are switched from media containing depolarizing levels of potassium, HK, to media containing repolarizing levels, LK, greater than 50% of the neurons undergo apoptosis within 24 h [9]. Though cell death begins at roughly 16 h, the neurons become impervious to rescue by 6 h following potassium deprivation [3]. Our lab and others have reported that apoptosis in granule neuron cultures can be prevented by the addition of survival factors to culture media including HK, IGF-1, cAMP, and lithium. The purpose of this report is to analyze the hypothesis that inactivation of GSK3β is a common downstream checkpoint in the survival promoting pathways initiated by these four survival factors.

2. Materials and methods

2.1. Materials

Unless otherwise noted, all materials were purchased from Sigma (St. Louis, MO). Phospho-Tyr216 GSK3β antibody was from BD Transduction Laboratories (San Jose, CA). All other antibodies were obtained from Cell Signaling Technologies (Beverly, MA). IGF-1 was purchased from Roche Biochemicals (Indianapolis, IN). ML-9, H-89, KN-62, SN-50, GSK3 inhibitor I, and TZDZ-8 were purchased from Calbiochem (La Jolla, CA).

2.2. Cell culture and treatments

Granule neuron cultures were obtained from dissociated cerebella of 7- to 8-day-old rats as described previously [9]. Cells were plated in basal Eagle's medium with Earles salts (BME) supplemented with 10% fetal bovine serum (FBS), 25 mM KCl, 2 mM glutamine (Gibco-BRL), and 100 μg/ml gentamicin on dishes coated with poly-L-lysine in 24-well dishes at a density 1.0×10^6 cells/well, 1.2×10^7 cells/60-mm dish, or 3.0×10^7 cells/100-mm dish. Cytosine arabinofuranoside (10 μM) was added to the culture medium 18–22 h after plating to prevent replication of nonneuronal cells. Cultures were maintained for 6–7 days prior to experimental treatments. For this, the cells were rinsed once and then maintained in low K^+ medium (serum-free BME medium, 5 mM KCl), high K^+ medium (serum-free BME medium, supplemented with 20 mM KCl), IGF-1 (50 ng/ml), forskolin (10 μM), or lithium (10 mM). Treatment of cultures with pharmacological inhibitors was initiated 30 min prior to rinsing and maintained throughout the subsequent incubation in specified medium unless mentioned otherwise. Pharmacological inhibitors were used at the following concentrations unless otherwise specified: H-89 at 10 μM, KN-62 at 50 μM, ML-9 at 20 μM, GSK3 inhibitor I at 10 μM, TZDZ-8 at 20 μM, SB-415286 at 30 μM.

2.3. Neuronal survival

Neuronal survival was quantified by the MTT assay as previously described [22]. Briefly, the tetrazolium salt MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide) was added to the cultures at a final concentration of 1 mg/ml, and incubation of the culture was continued in the CO_2 incubator an additional 30 min at 37 °C. The assay was stopped by adding lysis buffer [20% SDS in 50% *N*,*N*-dimethyl formamide, pH 4.7]. The absorbance was measured spectrophotometrically at 570 nm after an overnight incubation at room temperature. The absorbance of a well without cells was used as background and subtracted. Data are presented as mean ± standard deviation. Statistical analysis was performed using ANOVA and Student–Neuman–Keuls' test. Besides MTT assays, viability was also quantified using the fluorescein-diacetate method [9] and by 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) staining (which reveals apoptotic nuclei as condensed or fragmented). The results using these assays were similar to those obtained with the MTT assay.

2.4. Preparation of nuclear extracts

To isolate nuclei, cells were incubated for 30 min in buffer A containing 10 mM HEPES pH 7.9, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 50 mM NaF, 50 mM β-glycerophosphate, 5% glycerol, and 1× protease inhibitor cocktail. Following incubation, 1/10 the buffer A volume of 10% NP-40 was added and the mixture was vortexed for 30 s. Cytosolic extracts were obtained following brief centrifugation. The remaining nuclear pellet was resuspended in

buffer B containing 20 mM HEPES pH 7.9, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol, 1× protease inhibitor cocktail, and extracted on ice for 30 min, followed by micro-centrifugation at 16,000 × *g* for 10 min. The supernatants were collected as nuclear extracts. Concentrations of these nuclear extracts were determined by the Bradford method using reagents from Bio-Rad. Lysates were then used in Western blot analysis.

2.5. Western blotting

For whole cell lysates, the culture medium was discarded, the neurons washed twice with ice-cold phosphate-buffered saline (PBS), and lysed in lysis buffer (1% Triton, 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1× protease inhibitor mixture). Protein concentrations were measured using a Bradford protein assay kit (Bio-Rad), and equivalent amounts of protein were mixed with 6× SDS sample buffer (375 mM Tris–HCl [pH 6.8 at 25 °C], 12% SDS, 60% glycerol, 300 mM DTT, 0.012% bromophenol blue). Following heating at 95 °C for 5 min, proteins were subjected to SDS–polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene difluoride membrane (PVDF; Bio-Rad). After staining with Ponceau S to verify uniformity of protein loads/transfer, the membranes were analyzed for immunoreactivity. Incubation with primary antibodies was performed overnight at 4 °C and with secondary antibodies for 1 h at room temperature. Immunoreactivity was developed by enhanced chemiluminescence (ECL; Amersham) and visualized by autoradiography.

3. Results

3.1. GSK3β is activated during low potassium-induced neuronal apoptosis

As mentioned previously, cultures of CGNs switched from HK medium to medium containing LK result in morphological changes and appearance of biochemical markers indicative of apoptosis [9]. As shown in Fig. 1A, apoptosis-inducing LK treatment leads to dephosphorylation/activation of GSK3β at Ser9 as early as 2 h following potassium deprivation and continues through the time of commitment to cell death (6 h).

3.2. Forskolin, IGF-1, and lithium promote the phosphorylation and inactivation of GSK3β

We and others have established that LK-induced apoptosis of CGNs can be inhibited (and survival maintained) by IGF-1, cAMP, and lithium [10,23,24,29]. Other investigators have also reported the inactivation of GSK3β following IGF-1, cAMP, and lithium treatment [6,24,29]. To confirm and expand upon previous observations, we treated cultures of granule neurons with LK for 2 h and then applied survival stimuli in the form of forskolin (results in elevated levels of cAMP), IGF-1, and lithium to analyze the activation state of GSK3β. As shown in Fig. 1B, treatment of granule neuron cultures with forskolin, IGF-1, and lithium promotes the phosphorylation and inactivation of GSK3β at Ser9. Another site that is phosphorylated in GSK3β in nonneuronal cells is Tyr216 [32]. The phosphorylation status at Tyr216 can be regulated in response to certain extracellular stimuli [30] and phosphorylation at Tyr216 was previously reported to be associated with GSK3β activation. It is noteworthy however that more recent work has established that inhibition of GSK3β activity is regulated primarily by Ser9 phosphorylation and not by dephosphorylation of Tyr216 [32]. Not unexpectedly and in contrast to Ser9, treatment of neurons with all four survival factors did not alter the phosphorylation status of Tyr216 (Figs. 1C and D).

3.3. High potassium promotes the phosphorylation and inactivation of GSK3β

Although various papers have described the inactivation of GSK3β as a consequence of IGF-1, lithium, and cAMP signaling, none have illustrated the role of depolarization as a stimulus for the inactivation of GSK3β. As detailed in Fig. 1A, depriving neurons of potassium for 2 h leads to a decrease of pro-survival signaling, thus an activation of GSK3β. In Fig. 2, neurons were deprived of potassium for 2 h and then treated with HK for 15 and 30 min. Treatment of neurons with HK leads to a marked increase in the phosphorylation and thus inactivation of GSK3β.

3.4. Pharmacological inhibition of core components in survival signaling cascades causes apoptosis and is accompanied with activation of GSK3β

Small molecule inhibitors were used to inhibit core components in the survival signals initiated by HK, IGF-1, forskolin, and lithium. The goal was to test the hypothesis that inhibiting molecules downstream of the survival stimulus would result in apoptosis and that the observed apoptosis would associate with an increase in the activation in GSK3β. Treatment with the CaM Kinase II (CaMKII) inhibitor KN-62 demonstrated an ability to block membrane depolarization-mediated (HK) survival in cultures of sympathetic neurons [16,19]; thus, we used KN-62 in attempts to block HK-mediated survival of CGNs. As shown in Fig. 3A, treatment of granule neurons with HK, in the presence of KN-62, results in a 30%

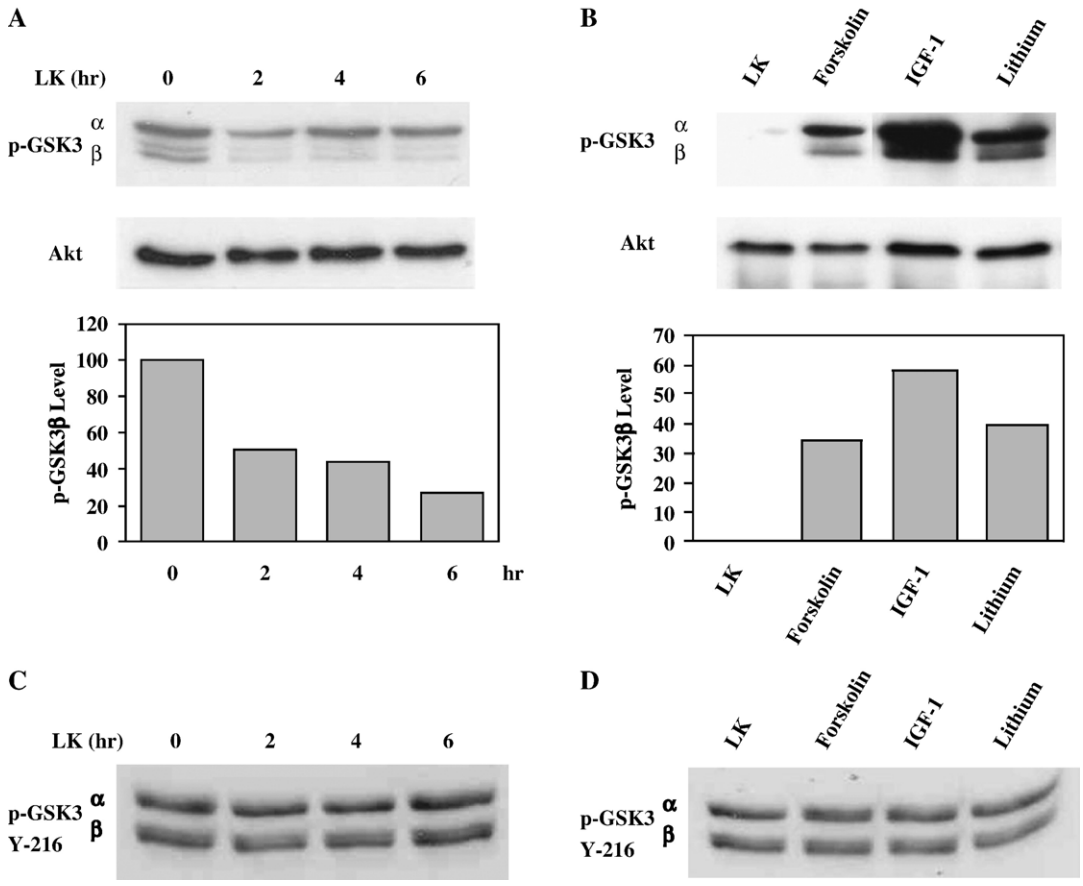

Fig. 1. GSK3β phosphorylation/activation in CGNs following treatment in LK, forskolin, IGF-1, and lithium. (A) Apoptosis-inducing low-potassium (LK) treatment leads to dephosphorylation/activation of GSK3β. Seven-day-old CGN cultures were switched to LK media for 2, 4, or 6 h. One culture was untreated (labeled LK 0 h). GSK3 phosphorylation at S-21/9 (Ser21 of GSK3α and Ser9 on GSK3β) was measured in lysates by Western blot using a phosphorylation-specific antibody. The same blot was then probed with an antibody for total levels of Akt to verify equal protein loading. Similar results were obtained in three separate experiments. The blot was quantified by densitometric analysis as the ratio of the loading control and plotted. (B) Forskolin, IGF-1, and lithium promote the phosphorylation/inactivation of GSK3β. Seven-day-old CGN cultures were switched to LK media for 2 h to cause activation of GSK3β. Lysates were obtained from neurons treated in either LK for 2 h or in LK for 2 h followed by stimulation with forskolin (10 μM), IGF-1 (50 ng/mL), or lithium (10 mM) for 30 min. GSK3 phosphorylation at S-21/9 was measured in lysates by Western blot using a phosphorylation-specific antibody. The same blot was then probed with an antibody for total levels of Akt to verify equal protein loading. Similar results were obtained in three separate experiments. The blot was quantified by densitometric analysis as the ratio of the loading control and plotted. Similar results were observed in other experiments. (C) Seven-day-old CGN cultures were switched to LK media for 2, 4, or 6 h. One culture was untreated (labeled LK 0 h). GSK3 phosphorylation at Y-216 was measured in lysates by Western blot using a phosphorylation-specific antibody. (D) Seven-day-old CGN cultures were switched to LK media for 2 h to cause activation of GSK3β. Lysates were obtained from neurons treated in either LK for 2 h or in LK for 2 h followed by stimulation with forskolin (10 μM), IGF-1 (50 ng/mL), or lithium (10 mM) for 30 min. GSK3 phosphorylation at Y-216 was measured in lysates by Western blot using a phosphorylation-specific antibody.

increase in apoptosis after 24 h relative to control HK cultures as measured by the MTT assay (similar results were obtained by DAPI staining). This inhibitor showed no effects on the survival mediated by IGF-1, cAMP, or lithium. Previously, we demonstrated that pharmacological inhibition of Akt with ML-9 in CGNs reduced the survival mediated by IGF-1 and lithium by as much as 50% after 24 h [5]. Furthermore, treatment of granule neuron cultures with forskolin leads to activation of adenylyl cyclase and thus an increase in cAMP levels in the cell [9]. Increased levels of cAMP lead to an increase in PKA activation and subsequent neuronal survival [13,24]; thus, we used H-89 to inhibit PKA in an attempt to prevent cAMP-mediated survival. Fig. 3B displays the effect of H-89 treatment on the survival of neurons directed by each of the survival factors. From Fig. 3B, it is apparent that H-89 impedes the survival controlled by forskolin, HK, and lithium after 24 h, as determined by the MTT assay (similar results were obtained by DAPI staining).

After establishing that pharmacological inhibition of core components of the survival signaling cascades could indeed prevent neuronal survival, we looked at the activation status of GSK3β in the conditions that were affected by pharmacological inhibition of survival signaling. Previously, we demonstrated that treatment of CGNs with IGF-1 in the presence of ML-9 leads to a decrease in the phosphorylation of GSK3β [5]. To explore this observation,

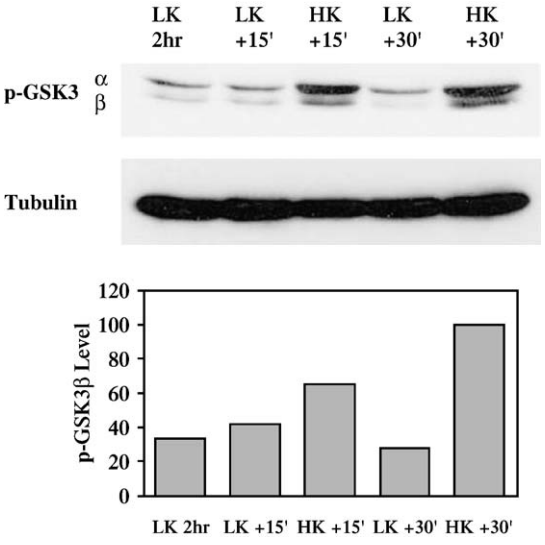

Fig. 2. HK promotes phosphorylation/inactivation of GSK3β. Seven-day-old CGN cultures were switched to LK media for 2 h to cause activation of GSK3β. The media were then supplemented with potassium (HK, 25 mM). Lysates were obtained from neuronal cultures either at 2 h in LK or following at an additional 15 and 30 min of LK or HK treatment. GSK-3 phosphorylation was measured in lysates by Western blot using a phosphorylation-specific antibody. The same blot was then probed with an antibody for tubulin to verify equal protein loading. Similar results were obtained in three separate experiments. The blot was quantified by densitometric analysis as the ratio of the loading control and plotted. Similar results were observed in other experiments.

cultures of granule neurons were switched to media containing LK for 2 h to lower survival signaling to basal levels. Prior to a 30 min stimulation with either HK, cAMP, or lithium, pharmacological inhibitors were added to the culture media for 30 min and continued throughout stimulation. Treatment with KN-62 in HK, treatment with ML-9 in lithium, and treatment with H-89 in HK, cAMP, and lithium generate a decrease in GSK3β phosphorylation, indicating an increase in activation of the enzyme as portrayed in Figs. 4A–C, respectively. These results suggest that apoptosis induced by pharmacological inhibition of core components in the survival signals initiated by HK, IGF-1, cAMP, and lithium may be a consequence of GSK3β activation.

3.5. Inhibition of NF-κB signaling leads to activation of GSK3β

NF-κB is a ubiquitously expressed and inducible transcription factor that is activated by all four survival factors, suggesting an important role for NF-κB in the promotion of neuronal survival [4,22]. Our lab has previously demonstrated that SN-50, a peptide that prevents the translocation of NF-κB to the nucleus, inhibits survival promotion by HK, IGF-1, and cAMP [22]. In addition, we documented that inhibition of NF-κB by SN-50 in IGF-1 signaling leads to decreased Akt activity [28]. This intriguing observation resulted in the discovery that, in some instances, Akt is a downstream target of NF-κB [28]. Thus, we investigated the possibility that promotion of neuronal apoptosis by inhibiting NF-κB in HK could lead to activation of GSK3β. Cultures of granule neurons were switched to LK for 2 h. Prior to stimulation with HK, SN-50 was added to cultures for 30 min. The results are depicted in Fig. 4A and show that GSK3β is activated by pharmacological inhibition of NF-κB signaling.

3.6. A small molecule inhibitor of GSK3β protects granule neurons from apoptosis

Recent studies have implicated GSK3β activity in the promotion of tau-associated neuropathologies including Alzheimer's disease (reviewed in [12,20]); thus, the generation of small molecule inhibitors of GSK3β could have therapeutic value. Recent screening programs have uncovered GSK3β inhibitory properties in six classes of

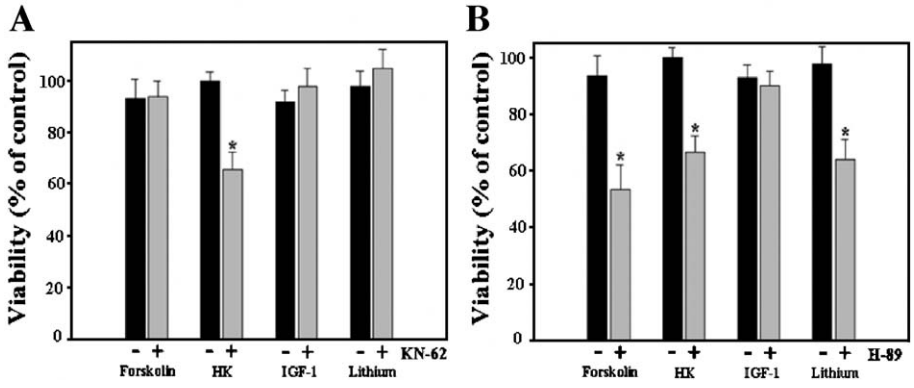

Fig. 3. Pharmacological inhibition of core components in survival signaling cascades selectively causes apoptosis. Cerebellar granule neurons were treated with forskolin (10 μM), HK (25 mM), IGF-1 (50 ng/mL), or lithium (10 mM) in the absence or presence of KN-62 (50 μM) (A) or H-89 (10 μM) (B). Cell viability was measured by the MTT assay 24 h later and is expressed as a percent of the HK control. Data represent the means ± SD from three separate experiments, each performed in duplicate. *Indicates significance $P < 0.001$. Similar results were obtained using DAPI staining to quantify apoptotic cells (condensed or fragmented nuclei).

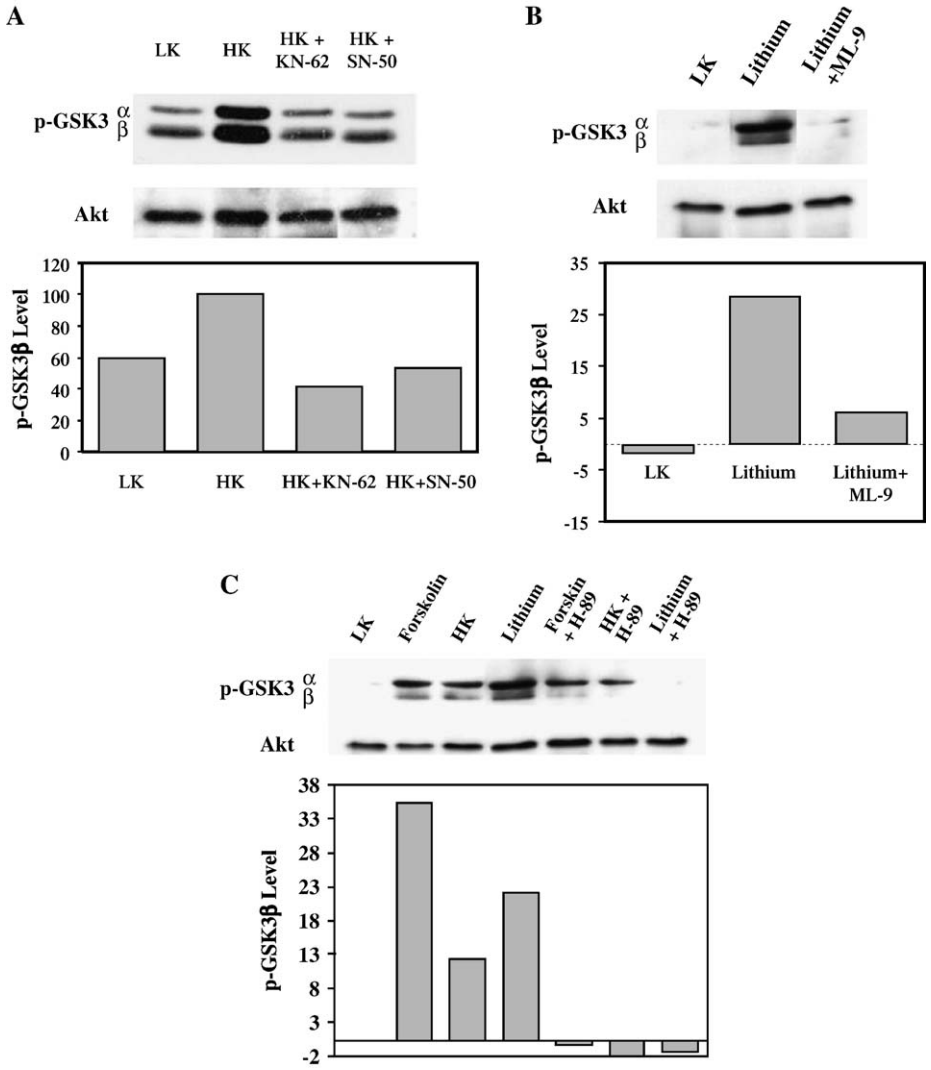

Fig. 4. Pharmacological inhibition of core components in survival signaling cascades leads to activation of GSK3β. Seven-day-old CGN cultures were switched to LK media for 2 h to cause activation of GSK3β. Lysates were obtained from neurons treated in either LK for 2 h or LK for 2 h followed by stimulation for 30 min with either HK (25 mM) (A), lithium (10 mM) (B), or forskolin (10 μM), HK, or lithium (C). Two cultures (A) were treated with either KN-62 (50 μM) or SN-50 (10 μM) for 30 min prior to stimulation with HK. One culture (B) was treated with ML-9 (20 μM) for 30 min prior to stimulation with lithium. Three cultures (C) were treated with H-89 (10 μM) for 30 min prior to stimulation with forskolin, HK, or lithium. GSK-3 phosphorylation was measured in lysates (A–C) by Western blot using a phosphorylation-specific antibody. Blots were then probed with an antibody for total levels of Akt to verify equal protein loading. Similar results were obtained in three separate experiments. The blot was quantified by densitometric analysis as the ratio of the loading control and plotted.

compounds: hymenialdisine, paullones, indirubines, maleidimides, muscarinic agonist, and thiadiazolidinones (reviewed in [27]). Moreover, thiadiazolidinones derivatives (TZDZ) inhibit GSK3β in a non-ATP competitive manner [26]. If inactivation of GSK3β is a common downstream checkpoint in the survival promoting pathways of cerebellar granule neurons, then inhibition of GSK3β activity by small molecule inhibitors should prevent apoptosis and represent a potential strategy against neurodegenerative diseases. We tested the ability of three of these compounds, GSK3 inhibitor I, TDZD-8, and SB-415286, shown in Fig. 5A, to prevent LK-induced apoptosis of cerebellar granule neurons. Fig. 5B illustrates the effects of GSK3β inhibitors in the prevention of LK-induced apoptosis. For these experiments, cultures were maintained in LK or LK supplemented with GSK3 inhibitor I, TDZD-8, or SB-415286. Viability of these cultures was measured 24 h later by MTT and depicted as percent of the HK control (similar results were obtained by DAPI staining). We attempted to rescue CGNs from LK-induced apoptosis with GSK3 inhibitor and TDZD-8 at concentrations ranging from 1 μM to 100 μM, but only SB-415286 prevented LK-induced apoptosis. Previous work has established that both GSK3 inhibitor I and TDZD are cell permeable [1]. This supports a previous report that demonstrated that both SB-415286 and a related compound, SB-216763, prevented apoptosis induced by inhibition of PI3-kinase pathway signaling in granule neurons [7].

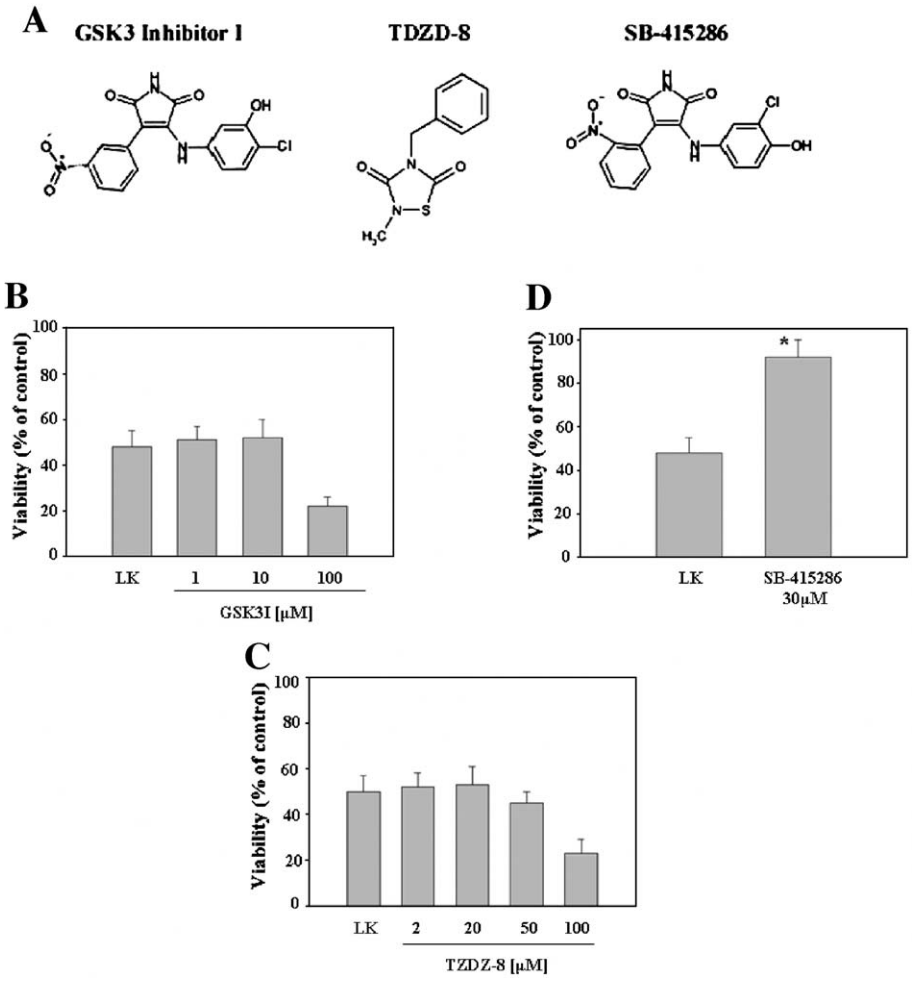

Fig. 5. GSK3 inhibitors and effect on LK-induced apoptosis. (A) Chemical structure of GSK3 inhibitor I, TDZD-8, and SB-415286. GSK3 inhibitor I and SB-415286 are structural analogues that inhibit GSK3β in vitro in an ATP competitive manner. TDZD-8 inhibits GSK3β in an ATP non-competitive manner. (B–D) Effects of GSK3 inhibitor I, TDZD-8, and SB-415286 on survival of CGN in LK. CGNs were treated with LK or LK supplemented with GSK3 inhibitor (1, 10, and 100 μM) (B), TDZD-8 (2, 20, 50, 100 μM) (C), or SB-415286 (30 μM) (D). Cell viability was measured by the MTT assay 24 h later and is expressed as a percent of the HK control. Data represent the means ± SD from three separate experiments, each performed in duplicate. *Indicates significance $P < 0.001$. Similar results were obtained using DAPI staining to quantify apoptotic cells (condensed or fragmented nuclei).

3.7. GSK-3 inhibitor I and TZDZ do not inhibit GSK-3β in CGNs

Given the inability of two out of three pharmacological inhibitors of GSK-3β to prevent apoptosis induced by LK, we performed experiments to assess the activity of GSK-3β in cells exposed to the inhibitors. The transcription factor β-Catenin is a substrate of GSK-3β, and active GSK-3β leads to decreased levels of β-Catenin in the nucleus [15]. Fig. 6 demonstrates that GSK-3 inhibitor I and TDZD-8 are unable to block the LK-induced, GSK-3β-directed downregulation of β-Catenin levels in nuclear extracts. On the other hand, treatment of granule neurons with LK supplemented with SB-415286 or lithium prevents the downregulation of β-Catenin. Although previous results have shown in vitro inhibition of GSK3β with GSK-3 inhibitor I and TDZD-8 [26,33], Figs. 6 suggests that these inhibitors are

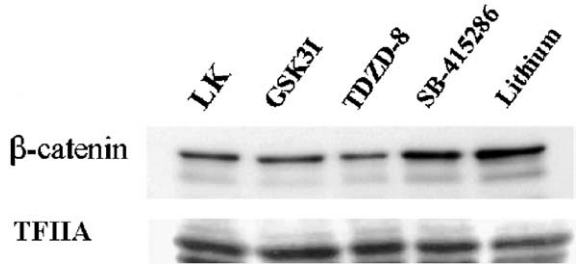

Fig. 6. GSK3 inhibitor I and TDZD-8 do not inhibit GSK3β activity in intact neurons. Cultures were treated for 4 h with LK or LK supplemented with GSK3 inhibitor I (10 μM), TDZD-8 (20 μM), SB-415286 (30 μM), or lithium (10 mM). Following incubation, nuclear lysates were obtained and subjected to Western blot analysis with a β-catenin antibody. Similar results were obtained in three separate experiments. The same blot was then probed with an antibody for levels of TFIIA to verify equal protein loading. Similar results were obtained in three separate experiments.

unsuccessful inhibitors of GSK-3β activity in intact neurons because they are unable to block signaling downstream of active GSK-3β.

4. Discussion

Many reports have implicated GSK3β in the promotion of apoptosis in a variety of paradigms in neuronal and non-neuronal cell types (reviewed in [14]). This report has confirmed and expanded upon previous observations regarding the role of GSK3β in the promotion of apoptosis of CGN. Our results suggest that GSK3β is a common downstream target in the survival promoted by HK, IGF-1, cAMP, and lithium. We demonstrated that the four survival factors regulate the activity of GSK3β, as measured by phosphorylation/inactivation specific antibody for GSK3. In addition, we blocked core components in the survival signaling pathways with pharmacological agents (H-89, ML-9, KN-62, and SN-50), resulting in apoptosis accompanied by a corresponding increase in GSK3β activity. Finally, pharmacological inhibition of GSK3 with SB-415286 prevented LK-induced apoptosis.

Although marketed as a PKA inhibitor, a recent report using purified kinases and synthetic substrates demonstrated that H-89 could inhibit additional kinases [8]. Although the concentration of H-89 (10 μM) employed should be selective for PKA, the ability of H-89 to inhibit the survival directed by HK and lithium could be due to inhibition of additional kinases. However, involvement of PKA in the survival directed by HK and lithium has not been excluded and thus warrants further investigation. More importantly, the apoptosis observed in pharmacological inhibition with H-89 in cAMP, HK, and lithium associated with increased activation of GSK-3β (decreased immunoreactivity of phosphorylated GSK3β) and thus emphasize the importance of GSK3β in the promotion of neuronal apoptosis.

We employed three pharmacological inhibitors of GSK3β in attempts to prevent LK-induced apoptosis of CGNs. Although GSK3 inhibitor I and SB-415286 are structural analogues, only SB-415286 was able to rescue CGNs from LK-induced apoptosis. We detected stabilization of β-catenin in CGNs treated with SB-415286 or lithium, but observed a decrease in expression of β-catenin in CGNs treated with LK, GSK3 inhibitor I, or TDZD-8. This suggests that although previous reports suggest inhibition of GSKβ in vitro with GSK3 inhibitor I or TDZD-8, they may not inhibit GSK3β activity in neurons for the purpose of rescuing CGNs from LK-induced apoptosis.

The observation that SB-415286 succeeded, while GSK3 inhibitor I or TDZD-8 failed to rescue CGNs from LK-induced apoptosis does not exclude the possibility that neuroprotection by SB-415286 is not exclusively due to the inhibition of GSK-3β. Abortive reentry into the cell cycle by post-mitotic neurons is a putative cause of apoptosis receiving increasing attention, and inhibition of cyclin-dependent kinases (CDKs) rescues neurons from apoptosis in a variety of paradigms (reviewed in [2]). The fact that GSK3 is closely related phylogenetically to CDKs supports the observation that many cell cycle inhibitors of CDK1, CDK2, and CDK5 also inhibit GSK3 (reviewed in [14,21]). Although GSK3 inhibitor I and TDZD-8 display no in vitro inhibition of CDK2 and CDK1, respectively [26,33], this has yet to be determined for SB-415286. If, in fact, SB-415286 does inhibit CDKs, combined inhibition of CDKs and GSK3β may be necessary and even beneficial to promoting the survival of neurons under degenerating conditions.

In summary, we have demonstrated that GSK3β is a convergent molecule in the regulation of CGN survival directed by HK, IGF-1, cAMP, and lithium. Moreover, GSK3β activity increases in LK-induced apoptosis and during pharmacological inhibition of core components of survival promoting pathways in CGNs. Finally, we demonstrated that pharmacological inhibition of GSK3β protects CGN from LK-induced apoptosis. Mounting evidence incriminates aberrant apoptosis as vital to the promotion of a variety of neurodegenerative conditions. Identifying the molecular mechanisms of cell death in primary cultures of neurons, thus, may help in the development of therapeutic targets. Our report supports the growing body of evidence suggesting that inhibition of GSK3β could have therapeutic potential in the treatment of neurodegenerative conditions.

Acknowledgments

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Phosphorylation of IκB-β Is Necessary for Neuronal Survival*

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Li Liu and Santosh R. D'Mello[1]

From the Department of Molecular and Cell Biology, University of Texas, Richardson, Texas 75083

Cerebellar granule neurons undergo apoptosis when switched from culture medium containing depolarizing levels of potassium (high potassium or HK) to nondepolarizing medium (low potassium or LK). We showed that in healthy neurons maintained in HK medium, IκB-β is phosphorylated at a novel site, Tyr-161. LK-induced neuronal apoptosis is accompanied by a decrease in the extent of IκB-β phosphorylation at this residue. Tyr-161 shares similarity to the consensus sequence for phosphorylation by the nonreceptor tyrosine kinases Abl and Arg. Arg phosphorylates Tyr-161 differentially *in vitro*, and LK treatment does cause a down-regulation of Arg activity. Moreover, treatment of neurons with two structurally distinct and highly selective Abl inhibitors, PD173955 and Gleevec, blocks HK-induced phosphorylation of IκB-β at Tyr-161 and induces neuronal apoptosis. Overexpression of wild-type IκB-β blocks LK-induced apoptosis, but this effect is abolished when Arg is pharmacologically inhibited. On the other hand, forced overexpression of IκB-β in which Tyr-161 is mutated inhibits survival in HK demonstrating the importance of this residue to neuronal survival. Phosphorylation of IκB-β enhances its association with p65/RelA causing an increase in NF-κB DNA binding activity. Our results identified IκB-β phosphorylation as a key event in neuronal survival and provided a mechanism by which this is mediated.

Apoptosis plays a critical role in the normal development of the nervous system by eliminating large numbers of superfluous neurons and ensuring proper neural connections. Aberrant apoptosis often occurs during adulthood leading to an unwanted loss of neurons such as that seen in neuropathological conditions, including Alzheimer, Parkinson, or Huntington disease and following ischemic stroke (reviewed in Ref. 1). Understanding the molecular mechanisms regulating apoptosis will therefore improve our understanding of neurodevelopment and will lead to the development of useful therapeutic strategies against neurodegenerative conditions.

One family of molecules that plays a pivotal role in the maintenance of neuronal survival in a variety of *in vivo* and *in vitro* experimental paradigms is NF-κB, a widely expressed transcription factor. Inhibition of NF-κB activity causes neuronal death in a variety of tissue culture paradigms of neurodegeneration (1–3). Reduced NF-κB activity has also been implicated in neurodegenerative diseases such as Alzheimer disease, Parkinson disease, and amyotrophic lateral sclerosis (reviewed in Ref. 4). In mammalian cells, there are five NF-κB proteins, p50, p52, p65 (RelA), RelB, and c-Rel, characterized by the presence of a conserved 300-amino acid Rel homology domain that is located toward the N terminus of the protein (reviewed in Refs. 5–8). Functional NF-κB is composed of homodimers and heterodimers of these proteins, typically p65:p50, which are held in the cytoplasm by association with members of the IκB protein family. Generally, but not always, activation of NF-κB is mediated by the phosphorylation of IκB proteins on two N-terminal serine or threonine residues by the IκB kinase (IKK)[2] complex, which contains the catalytic subunits IKKα and IKKβ and the regulatory IKK/NEMO protein. Phosphorylation by the IKK complex targets IκB for degradation via the ubiquitin-proteasome pathway. The released NF-κB thus translocates to the nucleus, where it binds to κB DNA motifs within the promoter regions of a variety of genes (6–8). In addition to nuclear translocation, more recent evidence indicates that maximal transcription activity of NF-κB requires protein-protein interaction and site-specific post-translational modifications, including phosphorylation and acetylation (6–8). Of the seven IκB family members, the best studied is IκB-α, which is phosphorylated by IKK at Ser-32 and Ser-36. Besides being a target of IKK, IκB-α can be phosphorylated at its C-terminal PEST domain by casein kinase-2 and DNA-PK (9–11). In contrast to IKK-mediated phosphorylation, however, IκB-α phosphorylation by these kinases does not cause its degradation (9–11). Another major member of the IκB family is IκB-β, which is phosphorylated by IKK at Ser-19 and Ser-23 (12). In comparison to IκB-α, relatively little is known about the functional significance of IκB-β. Although frequently assumed to be functionally interchangeable with IκB-α, more recent evidence indicates that IκB-β plays distinct roles within the cell (13, 14). Although association with IκB-β can inhibit NF-κB, in some situations, IκB-β can lead to increased NF-κB activity, although the precise mechanisms involved remain to be fully addressed (15, 16).

We have been studying the molecular mechanisms underlying neuronal survival using primary cultures of cerebellar granule neurons. These neurons undergo apoptosis when shifted from medium containing serum and depolarizing concentrations of potassium (high K^+ medium, HK) to medium containing low potassium (LK) (17). NF-κB is required for the survival of granule neurons by HK (2). Most interestingly, however, neither the levels nor intracellular distribution of the five NF-κB proteins nor those of IκB-α and IκB-β are altered in neurons primed to undergo apoptosis by LK treatment (2). We have reported previously that one factor involved in the down-regulation of NF-κB activity by LK treatment is a lowering interaction between p65 and cAMP-response element-binding protein-binding protein (CBP), an alteration that is associated with hyperphosphorylation (3).

In this study, we show that cerebellar granule neuron survival by membrane-depolarizing stimuli such as HK involves phosphorylation of IκB-β. HK-induced phosphorylation of IκB-β occurs at a novel site, Tyr-161. We also report that phosphorylated IκB-β associates with

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[1] To whom correspondence should be addressed: Dept. of Molecular and Cell Biology, University of Texas, 2601 N. Floyd Rd., Richardson, TX 75083. Tel.: 972-883-2520; Fax: 972-883-2409; E-mail: dmello@utdallas.edu.

[2] The abbreviations used are: IKK, IκB kinase; HK, high potassium; LK, low potassium; DAPI, 4,6-diamidino-2-phenylindole; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoresis mobility shift assay; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; EGFP, enhanced GFP; GST, glutathione *S*-transferase; EMSA, electrophoresis mobility shift assay; oligos, oligonucleotides; PB, phosphate buffer.

NF-κB and stimulates its DNA binding activity. In neurons primed to die by LK treatment, there is a reduction in IκB-β phosphorylation and binding activity of NF-κB. We present evidence that HK-induced phosphorylation of IκB-β is likely mediated by nonreceptor tyrosine kinases, Abl/Arg, molecules shown previously to regulate neuronal morphogenesis and axon guidance in the developing nervous system (18–22). Overexpression of wild-type IκB-β prevents LK-induced apoptosis in neurons, whereas addition of PD173955, a highly selective Abl/Arg inhibitor, abolishes the neuroprotection effect by IκB-β. Moreover, when IκB-β (Y161F) mutant is overexpressed in neurons, neuronal survival in HK is reduced. Our results also show that mutation on Tyr-161 of IκB-β decreases the interaction between IκB-β and p65, which is similar to the effect of LK treatment and HK treatment with the presence of Abl inhibitor. Thus, our results identify IκB-β phosphorylation as a key event in neuronal survival and provide a mechanism by which this is mediated.

EXPERIMENTAL PROCEDURES

Materials—Unless specified otherwise, all chemicals were purchased from Sigma. PD173955 was a kind gift from Dr. Bayard Clarkson (Memorial Sloan-Kettering Cancer Center, New York). CGP57148B (Gleevec) was obtained from Novartis Pharma AG (Switzerland). Both chemicals were dissolved in Me_2SO. Antibodies for p65 (sc-372), IκB-β (sc-945), IκB-α (sc-371), GST (sc-138), c-Abl (sc-23), Arg (sc-20708), and c-Jun (sc-1694) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Specific peptides for IκB-β (sc-945P) and p35 (sc-820P) were from Santa Cruz Biotechnology as well. Anti-α-tubulin (T-5168) was purchased from Sigma. Antibody for phosphotyrosine (9411) was purchased from Cell Signaling Technology (Beverly, MA). The above antibodies were used in Western blotting experiments. Anti-IκB-β, c-Abl, and Arg antibodies were also used in immunoprecipitation experiments. Anti-IκB-β and p65 antibodies were used in supershift experiments as well.

Cell Culture and Treatments—Granule neuron cultures were obtained from dissociated cerebella of 7–8-day-old rats as described previously (17). Cells were plated in Basal Eagle's Medium with Earle's salts (BME) supplemented with 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine (Invitrogen), and 100 μg/ml gentamycin on dishes coated with poly-L-lysine in 24-well dishes at a density 1.0×10^6 cells/well, 1.2×10^7 cells/60-mm dish, or 3.0×10^7 cells/100-mm dish. Cytosine arabinofuranoside (10 μM) was added to the culture medium 18–22 h after plating to prevent replication of non-neuronal cells. Unless indicated otherwise, cultures were maintained for 6–7 days prior to experimental treatments. For treatment, the cells were rinsed twice and then maintained in LK medium (serum-free BME medium, 5 mM KCl) or HK medium (serum-free BME medium, supplemented with 20 mM KCl). Unless indicated otherwise in the figure legends, treatment of cultures with pharmacological inhibitors was initiated 15 min prior to rinsing and was maintained through the subsequent incubation in LK or HK medium. The control cultures were treated with Me_2SO.

Plasmid Construction and Mutagenesis—pGEX-KG constructs containing full-length wild-type and the S19A/S23A mutation of IκB-β were the generous gifts from Dr. Richard B. Gaynor (Eli Lilly Co.). The truncation mutants were generated by PCR and cloned into pGEX-KG vector. Site-specific mutations in IκB-β were generated with the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA). The full-length IκB-β containing wild-type or mutations on S19A/S23A and Y161F, respectively, were subcloned into pEGFP-N1 (Clontech) vector at the N terminus of enhanced green fluorescence protein (EGFP). All plasmid constructs were confirmed by sequencing.

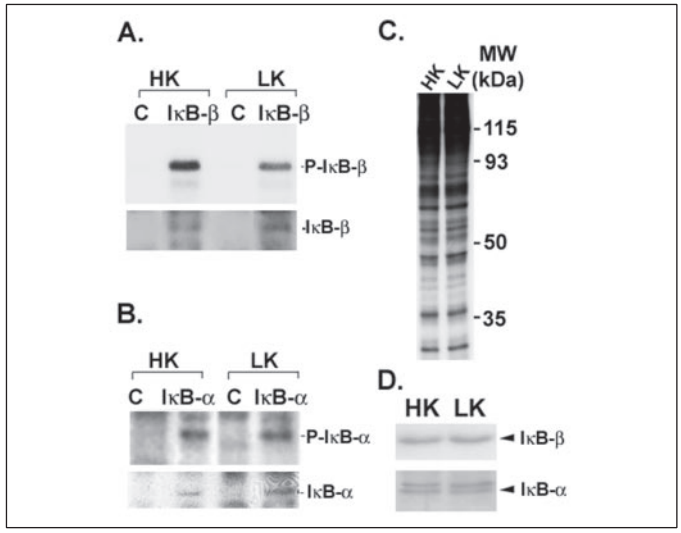

FIGURE 1. **HK enhances phosphorylation of IκB-β but not IκB-α *in vivo*.** Neurons were metabolically labeled with [^{32}P]orthophosphate in the presence of HK or LK medium for 4 h. Immunoprecipitation of IκB-β and IκB-α from the neuronal lysates was conducted with control (*C*) or IκB-β, IκB-α antibody. Immunoprecipitated samples were resolved on SDS-polyacrylamide gel. Part of the gel was dried and was followed by autoradiography. Another part was transferred to membrane for Western blot. *A*, autoradiograph of the gel (*top panel*) and Western blotting results with IκB-β antibody (*bottom panel*). *B*, autoradiograph of the gel (*top panel*) and Western blotting results with IκB-α antibody (*bottom panel*). *C*, autoradiograph of the ^{32}P-labeled neuronal lysates resolved on SDS-polyacrylamide gel. *D*, Western blot results to demonstrate levels of IκB-β (*top*) and IκB-α (*bottom*) in lysates prepared from HK- or LK-treated neurons.

Expression of Bacterially Produced GST-IκB-β Proteins—The pGEX-KG-IκB-β constructs were transformed into *Escherichia coli* BL21 (DE3) PlyS (Promega, Madison, WI). Cultures (250 ml) of *E. coli* were grown to an absorbance at 600 nm of 0.4 to 0.6 and induced with 0.4 mM isopropyl β-D-thiogalactopyranoside for 3 h to induce the expression of the GST fusion protein. Cells were pelleted, resuspended in buffer A (20 mM HEPES, pH 7.9, 400 mM NaCl, 5 mM dithiothreitol (DTT), 10% glycerol, 0.1 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF)), mildly sonicated, and centrifuged. The supernatant was incubated with 0.5 ml of glutathione-agarose beads (G-4510, Sigma) for 2 h at 4 °C. The matrix was washed four times with buffer A and resuspended in buffer A for GST pull-down assay. To obtain soluble fusion proteins, the beads were further washed two times with 5 ml each of buffer B (50 mM HEPES, pH 8.3, 150 mM NaCl, 0.5% Nonidet P-40, 5 mM DTT, 1 mM PMSF). The fusion proteins were eluted off beads by 10 mM glutathione in buffer B at 37 °C for 30 min and stored at −80 °C.

Neuronal Survival—Neuronal survival was assessed by staining neurons with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) to show the apoptotic characteristics of nuclei after treatment. Briefly, cells growing on coverslips were washed in 0.1 M PB, pH 7.4, and fixed in 4% paraformaldehyde in PB, pH 7.4, for 15 min at room temperature. Cells were then washed once with PB for 5 min at room temperature followed by staining with DAPI solution (0.5 μg/ml) for 10 min at room temperature. After staining, cells were washed again with PB for 5 min. Coverslips were then mounted on slides. Cells were visualized under a fluorescence microscope, and the number was counted. Results from at least three separate experiments were statistically analyzed.

Preparation of Nuclear and Cytosolic Extracts—To obtain cytoplasmic proteins, cells were washed with cold phosphate-buffered saline, pH 7.2, resuspended in buffer containing 10 mM HEPES, pH 7.9, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 50 mM NaF, 1 mM sodium orthovanadate, 50 mM β-glycerophosphate, 5% glycerol, and protease inhibitor mixture (Roche Applied Science), and incubated on ice for 15 min. At

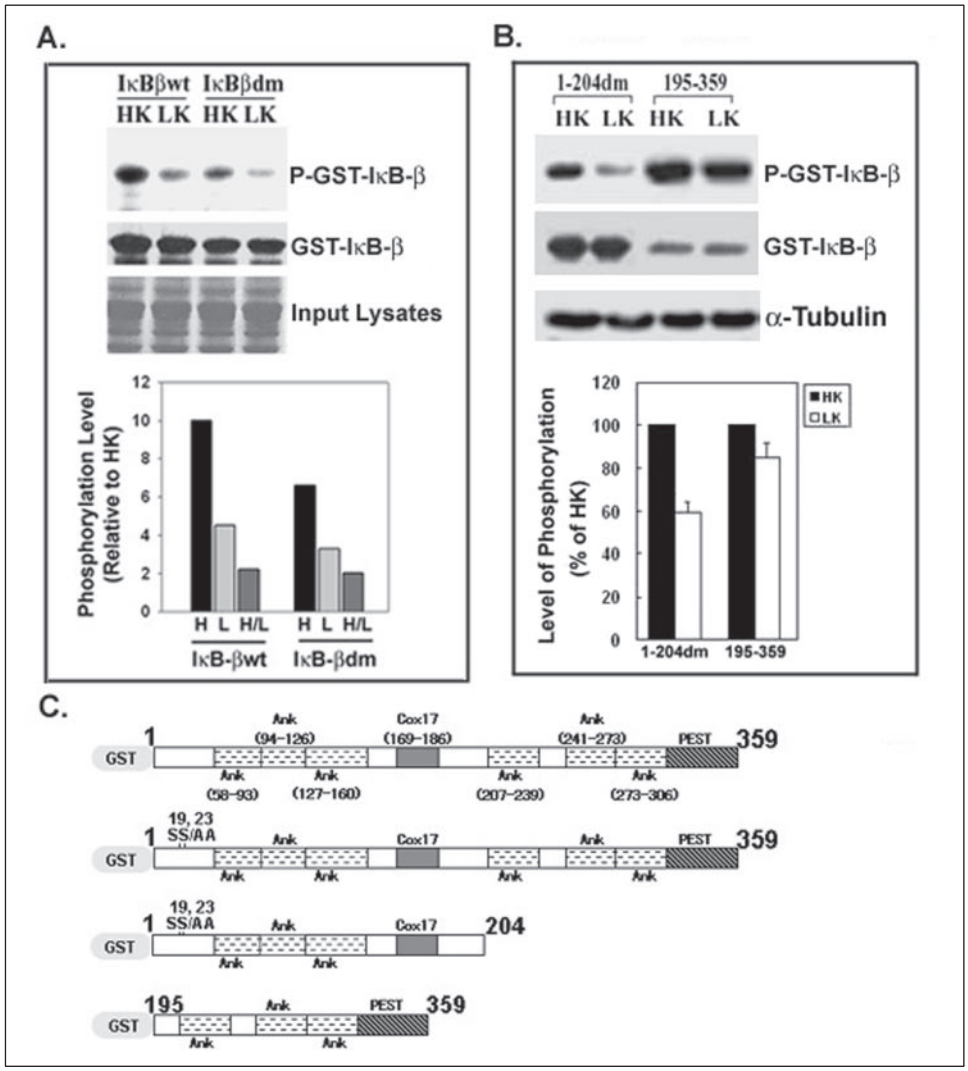

FIGURE 2. **Differential phosphorylation of GST-IκB-β proteins by cellular extracts from HK- or LK-treated neurons.** Bacterially expressed GST-IκB-β (*wt*), (S19A/S23A, shown as *dm*), C-terminally truncated (*1–204dm*), and N-terminally truncated (*195–359*) proteins were bound to glutathione-agarose beads. These beads were incubated with cellular extracts prepared from cerebella granular neurons treated for 6 h under HK or LK conditions. *In vitro* kinase assay was conducted with [γ-^{32}P]ATP. *A*, an autoradiographic image (*top panel*) for phosphorylation of full-length GST-IκB-β. Coomassie Blue staining of GST-IκB-β proteins that were used as substrates are shown in the *2nd panel*. Coomassie Blue staining image for supernatants after GST pull-down assay are shown in the *3rd panel*. Levels of phosphorylated GST-IκB-β (*top panel*) were quantified by using ImageQuant and normalized with levels of both IκB-β protein (*2nd panel*) and input proteins (*3rd panel*). The value for phosphorylation of wild-type (*wt*) GST-IκB-β at HK was set to 10, and others were valued based on the relative level to the P-GST-IκB-β at HK. The *H/L bars* refer to the ratio of P-GST-IκB-β at HK *versus* LK (*bottom panel*). *B*, representative autoradiographic image for truncated forms of GST-IκB-β (*top panel*), Western blot with GST antibody (*2nd panel*), and Western blot with α-Tubulin antibody (*3rd panel*). Levels for P-GST-IκB-β were quantified and normalized with GST-IκB-β and α-tubulin levels. The values for phosphorylation of both GST-IκB-β proteins at HK were set at 100, and others were valued based on the relative levels to the P-GST-IκB-β at HK. Results from three separate experiments were analyzed and shown as mean ± S.E. (*4th panel*). *C*, schematic structure of GST-IκB-β fusion proteins is as follows: wild-type IκB-β (*1st lane*), IκB-β with mutations of serine residues 19 and 23 to alanine (*2nd lane*, referred as IκB-βdm), IκB-βdm with residues 205–359 deleted (*3rd lane*, 1–204dm), and IκB-β with residues 1–194 deleted (*4th lane*). The positions of the ankyrin (*Ank*) repeats, the PEST domain, and serine and/or tyrosine residues that were mutated are shown.

the end of incubation, 1/20 volume of 10% Nonidet P-40 was added. Cells were vortexed for 30 s and then subjected to centrifugation for 30 s at 14,000 rpm. Supernatants were collected as cytosolic proteins.

Nuclei from neurons were resuspended in buffer containing 20 mM HEPES, pH 7.9, 50 mM KCl, 420 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, protease inhibitor mixture and extracted on ice for 30 min, followed by centrifugation at 14,000 rpm for 5 min at 4 °C. The supernatants were collected as nuclear extracts. Protein concentrations of the cellular proteins were determined by the Bradford assay using Bio-Rad reagent.

Western Blots—50 μg of cellular extracts or protein complex pulled down by GST fusion protein or antibodies were resolved on SDS-polyacrylamide gels. The proteins were then transferred to nitrocellulose membranes. Membranes were blocked and incubated with the various antibodies mentioned previously. After washes, membranes were incubated with horseradish peroxidase-conjugated secondary antibody. Following the incubation, membranes were washed extensively and developed with ECL luminal reagent (Amersham Biosciences). The image was captured on x-ray film. Data were quantified using ImageQuant software (Amersham Biosciences).

Immunoprecipitation—100–200 μg of cellular lysates from treated neurons was incubated with control and specific antibodies (1 to 2 μg) on ice for 30 min. Protein A/G-agarose beads were added to the mixture, and further incubation was carried out at 4 °C overnight. After incubation, beads were pelleted by centrifugation followed by three washes with buffer containing 10 mM HEPES, pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, and protease inhibitor mixture. The beads were then used in variety of experiments. Supernatants from the immunoprecipitation were collected and resolved on SDS-polyacrylamide gels for Western blotting of α-tubulin antibody.

Analysis of Phosphorylation on Endogenous IκB-β—100-mm dishes of 7–8-day-old neurons were washed twice with warm, phosphate-free DMEM (Invitrogen) and incubated in phosphate-free DMEM containing 20 mM KCl overnight. The cultures were then incubated for 6 h in medium containing [^{32}P]orthophosphate (MP Biomedicals, Irvine, CA) with the indicated treatment. After being lysed in ice-cold RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 50 mM NaF, 30 mM β-glycerophosphate, 1 mM EDTA, protease inhibitors mixture), the lysates were subjected to immunoprecipitation as described, and the proteins were separated on SDS-polyacrylamide gel. After electrophoretic transfer to nitrocellulose membrane, labeled proteins were visualized by autoradiography. Results were obtained by scanning on Storm860 (Amersham Biosciences). Data were quantified using ImageQuant software (Amersham Biosciences).

GST Pull-down and in Vitro Kinase Assay—GST-IκB-β proteins were bound to glutathione-agarose beads. The beads were incubated with whole cell lysates prepared from HK- and LK-treated neuronal

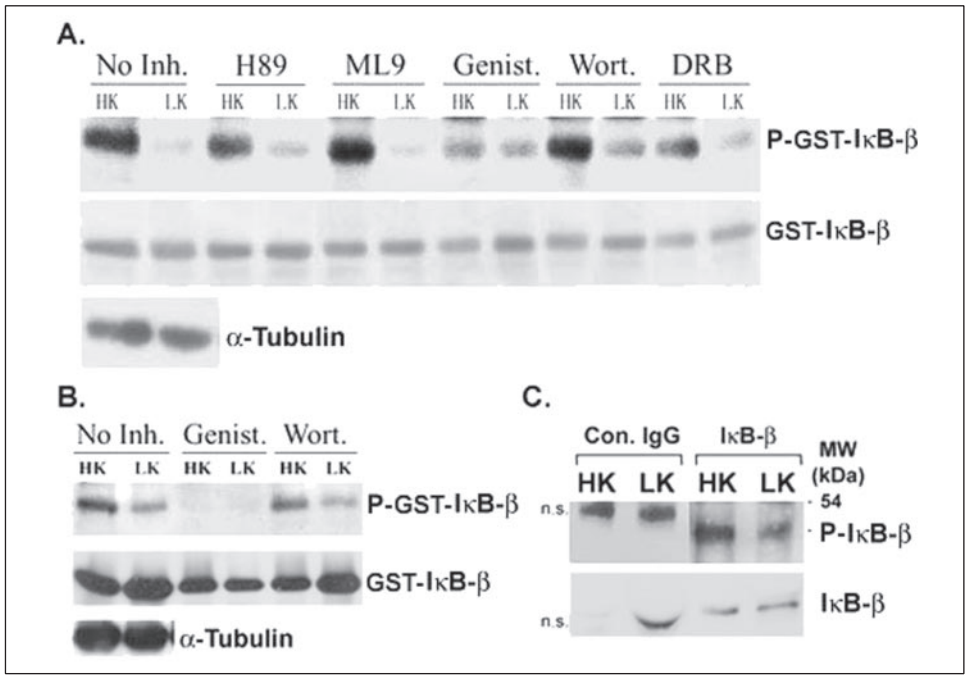

FIGURE 3. ***In vitro* inhibition of kinase activity by protein kinase inhibitors and *in vivo* phosphorylation of IκB-β on tyrosine residue(s).** GST-IκB-β mutant (1–168 $^{S19A/S23A}$) bound to glutathione-agarose beads was incubated with cellular extracts prepared from HK- or LK-treated neurons (6 h). GST pull-down and *in vitro* kinase assays were then conducted. Various protein kinase inhibitors were added during the kinase assay. Concentrations for these inhibitors are as follows: *H89* (10 μM), *ML-9* (40 μM), genistein (*Genist.*) (50 μM), wortmannin (*Wort.*) (400 nM), and 5,6-dichlorobenzimidazole riboside (*DRB*) (50 μM). *No Inh.*, no inhibitor. *A*, phosphor-GST-IκB-β (*top panel*) and total GST-IκB-β protein (Western blot with anti-GST, *middle panel*). Level of α-tubulin in the input lysates (*bottom panel*). *B*, similar experiment was conducted with nonradioactive ATP. Western blot with Tyr(P) antibody was performed on the samples. Tyrosine-phosphorylated IκB-β is shown in the immunoblot with anti-Tyr(P), *top panel*. IκB-β protein used in the assay is shown in the immunoblot with anti-GST, *middle panel*. Level of α-tubulin in the input cellular extracts is shown in the *bottom panel*. *C*, lysates from HK- or LK-treated neurons (6 h) were immunoprecipitated with control IgG (*Con. IgG*) or IκB-β antibody. Immunoprecipitated proteins were resolved on SDS-polyacrylamide gel. Western blot was conducted with Tyr(P) (*top panel*, *n.s.*, nonspecific) and IκB-β (*bottom panel*) antibody, respectively.

cultures. The whole cell lysates were generated from cultures plated in 100-mm dishes (30×10^6 cells/dish) and lysed in a volume of 250 μl. The kinase assay was performed in the kinase reaction buffer containing 20 mM HEPES, pH 7.9, 100 mM KCl, 5% glycerol, 0.2 mM EDTA, 4 μM ATP, 10 μCi of [γ-^{32}P]ATP, 5 mM NaF, 1 mM Na_3VO_4, 40 μM $MgCl_2$, protease inhibitor mixture at 30 °C for 30 min. The beads were pelleted by brief centrifugation. After addition of 4× SDS sample buffer to the pellet, the samples were heated at 95 °C for 5 min. The proteins were resolved on 10% SDS-polyacrylamide gels followed by autoradiography. Results were obtained by scanning with Storm860. Data were quantified using ImageQuant.

Gel Electrophoresis Mobility Shift Assay (EMSA)—Nuclear extracts were incubated with 0.1 pmol of ^{32}P-labeled double-stranded κB-binding site oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGG-3′), SP1-binding site oligonucleotide (5′-ATTCGATCGGGGCGGGGC-GAGC-3′), or TFIID-binding site oligonucleotide (5′-GCAGAG-CATATAAGGTGAGGTAGGA-3′) in buffer containing 1 μg of poly[d(I·C)], 1 μg of bovine serum albumin, 10 mM HEPES, pH 7.9, 0.5 mM DTT, 0.1 mM EDTA, 60 mM KCl, 0.2 mM PMSF, 5 mM $MgCl_2$, and 12% glycerol at room temperature for 15 min. Samples were analyzed by 5% native PAGE followed by autoradiography. For competition experiments, nuclear extracts were incubated with nonradiolabeled κB oligonucleotide (5′-TCGACAGAGGGGACTTTCCGAGAGGCT-3′) or AP1 oligonucleotide (5′-CGCTTGATGAGTCAGCCGGAA-3′) for 15 min on ice prior to the addition of ^{32}P-labeled double-stranded κB-binding site oligonucleotide. For supershift experiments, specific antibody was incubated with nuclear extract for 15 min on ice followed by further incubation with ^{32}P-labeled double-stranded κB-binding site oligonucleotide.

Analysis of DNA Fragmentation—The neurons (60-mm plate) were treated with various reagents. After 24 h, the cells were harvested and washed with cold phosphate-buffered saline. The cell pellets were resuspended in 0.1 ml of lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.2% Triton X-100) and incubated on ice for 20 min. Lysed cells were centrifuged at 12,000 rpm for 10 min at 4 °C. The DNA was precipitated from the supernatants by adding a 0.1 volume of 5.0 M NaCl and a 0.5 volume of isopropyl alcohol. After incubation at −20 °C, the samples were centrifuged at 12,000 rpm for 10 min. The resulting pellets were resuspended in 50 μl of Tris/EDTA buffer containing proteinase K (300 μg/ml) and RNase A (100 μg/ml) and incubated for 30 min at 50 °C. The samples were subsequently analyzed on a 1.5% agarose gel in 1× Tris borate/EDTA buffer.

Transient Transfection—Transient transfection of granule neurons was carried out as outlined by Koulich *et al.* (2). Briefly, on day 5 after plating, 10 μg/well of DNA was precipitated by calcium phosphate method at room temperature for 30 min and added dropwise to cultures that has been washed once with DMEM without L-glutamine, serum, and antibiotics (transfection DMEM). Cells were incubated in DMEM at 37 °C for 75–90 min followed by two washes with the medium. The transfection DMEM was then replaced with the original medium. The next day, cells were treated in HK or LK or with the addition of PD173955 (2 μM) for 24 h. Cells were then fixed, and DAPI staining was performed. Expression of EGFP and the DAPI-stained nucleus was visualized under a fluorescence microscope. Transfected cells were determined by merging images from GFP and UV filters.

RESULTS

Phosphorylation of IκB-β but Not IκB-α Is Reduced during Neuronal Apoptosis—Cerebellar granule neurons undergo apoptosis when switched from HK medium to LK medium. Although cell death begins at about 16 h, previous studies have shown that commitment to death occurs within 6 h after the switch to LK medium (23–26). As shown in Fig. 1*A*, IκB-β is phosphorylated in HK medium, and LK treatment leads to a reduction in the level of IκB-β phosphorylation. The reduction in IκB-β phosphorylation is detectable as early as 4 h after LK treatment, suggesting that it is causally involved in the induction of neuronal apoptosis (Fig. 1*A*). In contrast to IκB-β, the phosphorylation level of IκB-α is similar in HK or LK medium (Fig. 1*B*). The overall pattern of protein phosphorylation (Fig. 1*C*) and protein levels of both IκB-α and IκB-β are not altered by the treatment with HK and LK conditions (Fig. 1*D*), indicating that the reduced phosphorylation of IκB-β is specific.

HK-induced IκB-β Phosphorylation Occurs at Site(s) Independent of IKK—It is known that IKK phosphorylates IκB-β at Ser-19 and Ser-23. We examined whether HK-induced IκB-β phosphorylation was medi-

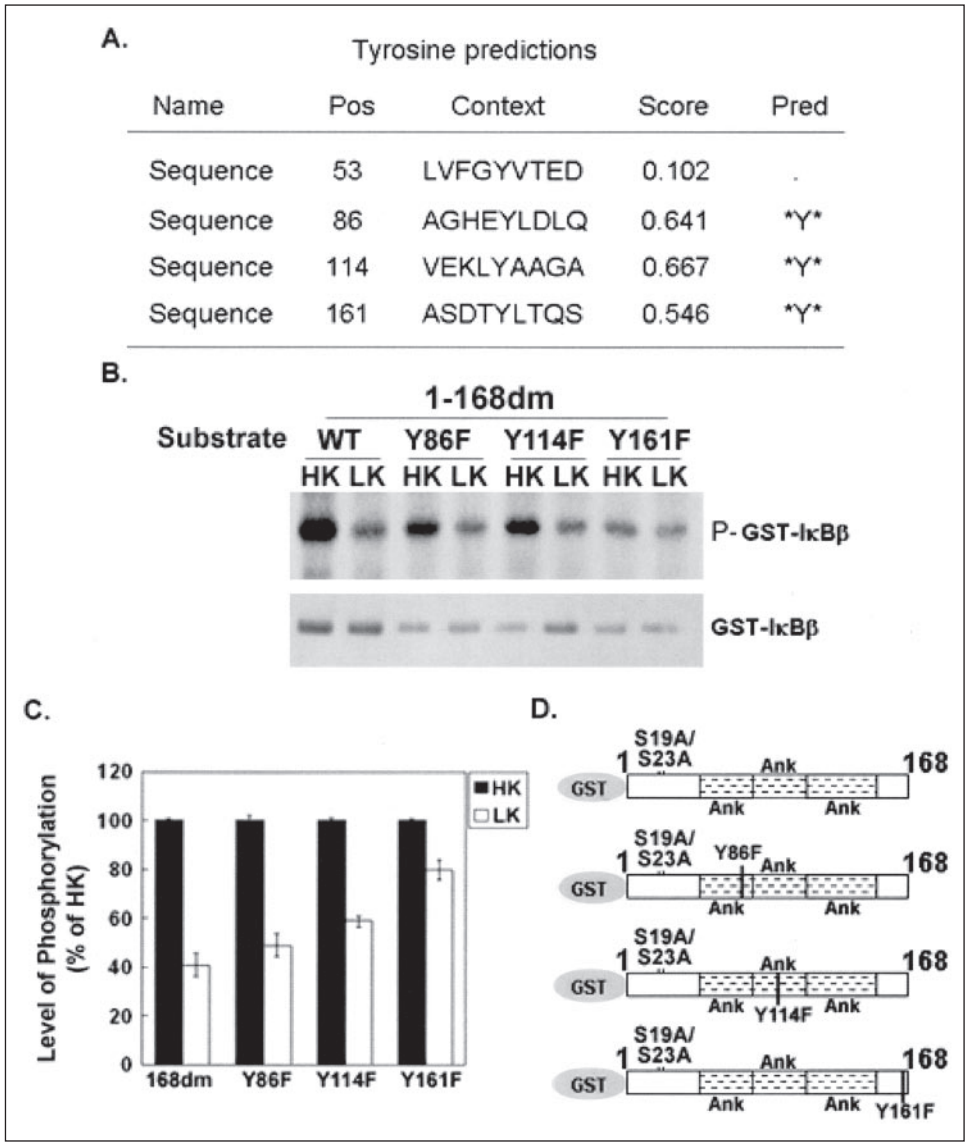

FIGURE 4. **Identification of a tyrosine residue that is phosphorylated differentially in HK or LK conditions.** *A*, tentative tyrosine residues in IκB-β (1–168 $^{S19A/S23A}$) predicted by NetPhos 2.0 Sever (www.cbs.dtu.dk/services/NetPhos/) that can be phosphorylated. Site-specific mutagenesis was conducted on pGEX-KG IκB-β (1–168 $^{S19A/S23A}$) on Tyr-86, Tyr-114, and Tyr-161, respectively. These mutated IκB-β constructs were expressed into proteins. GST-IκB-β proteins bound with glutathione-agarose beads were incubated with cytosolic extracts prepared from HK- and LK-treated neurons (6 h), followed by *in vitro* kinase assay with [γ-^{32}P]ATP. *Pos*, position; *Pred*, predicted. *B*, the *top panel* shows a representative image of the kinase assay. The *bottom panel* shows Western blot results with GST antibody. *WT*, wild type. *C*, quantified results from three separate experiments are shown as mean ± S.E. All the results were obtained by normalizing P-GST-IκB-β levels with both substrate and input cellular protein levels. Levels of P-GST-IκB-β at HK are set to 100. Others are valued based on the relative level to the P-GST-IκB-β at HK. *D*, schematic structure of GST-IκB-β fusion proteins used. For IκB-βdm truncated from residues 169 to 359 (*1st lane*), this construct with mutation of tyrosine residue 86 to phenylalanine (*2nd lane*), tyrosine residue 114 to phenylalanine (*3rd lane*), and tyrosine residue 161 to phenylalanine (*4th lane*). *Ank*, ankyrin.

ated by IKK by using a mutant GST-IκB-β construct in which both Ser-19 and Ser-23 were mutated (GST-IκB-β$^{S19A/S23A}$, see Fig. 2*C*). This construct can therefore not be phosphorylated by IKK. As shown in Fig. 2*A*, although the level of phosphorylation on GST-IκB-β in both HK and LK was somewhat reduced, the difference in phosphorylation between HK and LK extracts seen with wild-type GST-IκB-β was also observed in the double mutant. The same result was obtained using a C-terminally truncated form of GST-IκB-β, which lacks the two target sites of IKK (Fig. 2*B*). The similarity in the difference of phosphorylation elicited in HK and LK medium between wild-type IκB-β and IκB-β$^{S19A/S23A}$ or the C-terminally truncated mutant suggests that the differential phosphorylation on IκB-β during neuronal apoptosis happens on residue(s) that are not sensitive to IKK.

Mapping Apoptosis-regulated Phosphorylation Site—To map the site within IκB-β that is differentially regulated during LK-induced apoptosis, several deletion constructs were generated and used in *in vitro* kinase assays with extracts from HK- and LK-treated neurons (Fig. 2*C*). As shown in Fig. 2*B*, GST-IκB-β fragments spanning residues 1–204$^{S19A/S23A}$ displayed differential phosphorylation similar to that seen with the full-length GST-IκB-β protein. In contrast, fragment spanning residues 195–359 were not differentially phosphorylated. Another fragment spanning residues 1–168$^{S19A/S23A}$ also displayed elevated phosphorylation level in HK compared with LK (data not shown). These results localize the HK-induced phosphorylation site to the region spanning residues 1–168 of IκB-β.

To determine whether the apoptosis-regulated phosphorylation site was a Tyr residue or a Ser/Thr residue, we used genistein, a broad spectrum pharmacological inhibitor of tyrosine kinases. Addition of genistein to the *in vitro* kinase assay abolished the differential phosphorylation of GST-IκB-β (Fig. 3*A*), suggesting that the HK-induced phosphorylation occurred at a Tyr residue. In comparison, other inhibitors, such as the phosphatidylinositol 3-kinase inhibitor wortmannin, the Akt inhibitor ML-9, and the casein kinase-II inhibitor 5,6-dichlorobenzimidazole riboside, did not affect the differential phosphorylation pattern (Fig. 3*A*). Western blot analysis of GST-IκB-β in the *in vitro* kinase assay with a phosphotyrosine antibody also showed increased immunoreactivity in HK compared with LK, providing additional evidence that the HK-induced phosphorylation of IκB-β occurred at Tyr residue(s) (Fig. 3*B*). The higher level of phosphotyrosine immunoreactivity was abolished with the addition of genistein (Fig. 3*B*). To confirm that tyrosine phosphorylation on I κB-β protein also occurred *in vivo*, immunoprecipitation was performed with IκB-β antibody following by Western

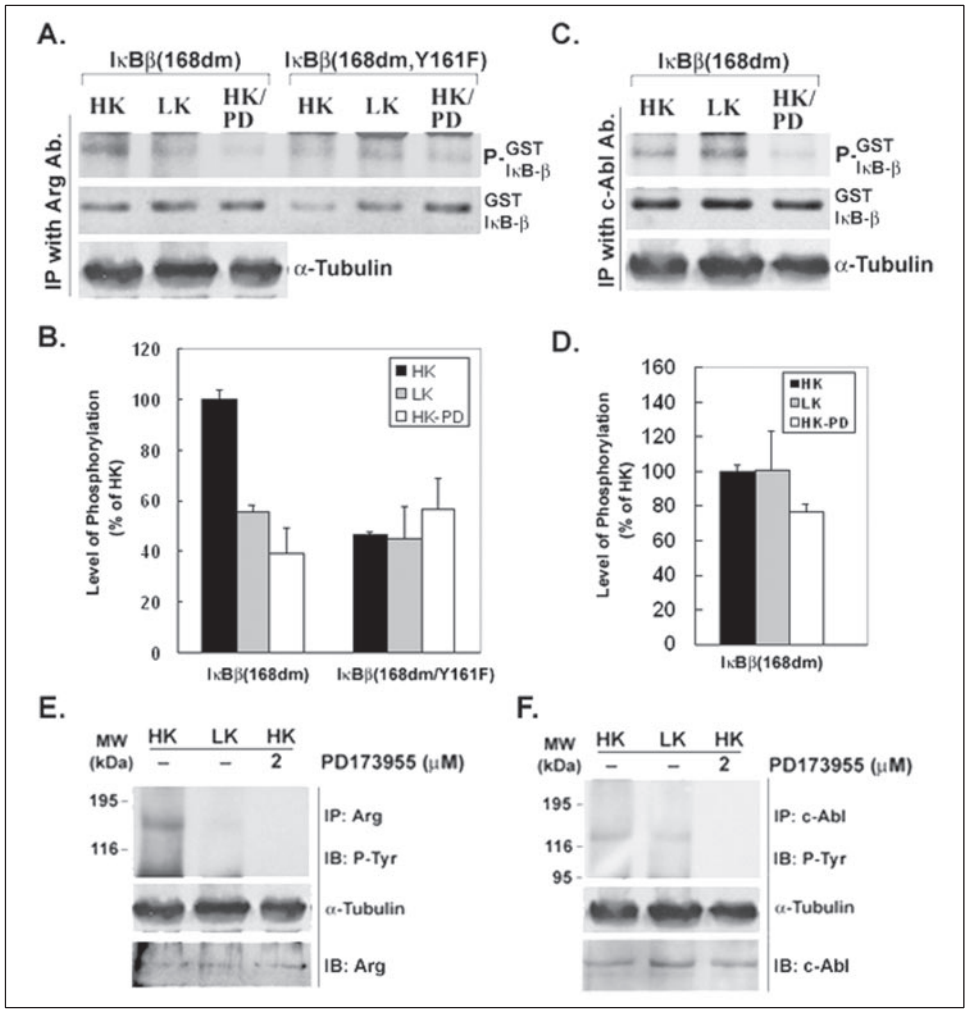

FIGURE 5. **PD173955 inhibits Abl/Arg-mediated phosphorylation on Tyr-161 of GST-IκB-β.** Immunoprecipitation (*IP*) was performed using anti-Arg or c-Abl antibody (*Ab*) with cellular extracts prepared from neurons treated for 6 h under HK, LK, or HK with PD173955 (2 μM) conditions. *In vitro* kinase assay was conducted on immunoprecipitated complex with GST-IκB-β(1–168dm) or GST-IκB-β(1–168dmY161F) as substrate. *A*, results relating to immunoprecipitation with Arg antibody are shown as follows: autoradiograph for phosphorylation of GST-IκB-β (*top panel*), Western blot with GST antibody (*middle panel*), and Western blot with α-tubulin antibody on the samples of supernatants from immunoprecipitation (*bottom panel*). *B*, quantified result for *A* is shown. Levels of P-GST-IκB-β were normalized with levels of input GST-IκB-β and α-tubulin. Values of P-GST-IκB-β at HK are set to 100. Others are valued based on the relative level to the P-GST-IκB-β at HK. The results are expressed as the mean ± S.D. of three separate experiments. *C*, results relating to immunoprecipitation with c-Abl antibody are shown as follows: autoradiograph for phosphorylation of GST-IκB-β (*top panel*), Western blot with GST antibody (*middle panel*), Western blot with α-tubulin antibody on the samples of supernatants from immunoprecipitation (*bottom panel*). *D*, quantified result for *C* is shown. Procedure for data processing is the same as mentioned above in *B*. *E*, the membrane used in *A* is reprobed with the Tyr(P) antibody (*top*) and Arg antibody (*bottom*). *IB,* immunoblot. *F,* the membrane used in *C* is reprobed with Tyr(P) antibody (*top*) and c-Abl antibody (*bottom*).

blotting with Tyr(P) antibody. Results in Fig. 3*C* reveal that a higher level of Tyr(P) immunoreactivity was detected with the HK-treated neuronal lysate, whereas Western blotting results with the IκB-β antibody show similarity to total IκB-β immunoreactivity. These *in vivo* and *in vitro* results suggest that HK-induced phosphorylation on IκB-β happens on tyrosine residue(s).

Analysis of the amino acid sequence of IκB-β in the region between residues 1 and 168 revealed four tyrosine residues, Tyr-53, Tyr-86, Tyr-114, and Tyr-161, that are conserved among rat, mouse, and human IκB-β proteins. Among them, Tyr-86, Tyr-114, and Tyr-161 are identified by NetPhos2.0(www.cbs.dtu.dk/services/NetPhos/) as potential phosphorylation sites (Fig. 4*A*). We investigated whether any of these residues represented the apoptosis-regulated phosphorylation site by using GST-IκB-β in which each of these three sites were mutated in *in vitro* kinase assays (Fig. 4*D*). As shown in Fig. 4, *B* and *C*, only mutation of Tyr-161 reduced the extent of phosphorylation seen with HK extracts.

Apoptosis-regulated Phosphorylation of IκB-β Is Mediated by Arg in Vitro—Tyr-161 resides within the consensus sequence for phosphorylation by the nonreceptor tyrosine kinase Abl (analyzed by ScanSite server at scansite.mit.edu). Two Abl kinases are expressed in mammalian cells, c-Abl and Arg. As a step toward examining whether these kinases were responsible for HK-induced IκB-β phosphorylation, we immunoprecipitated c-Abl or Arg from neuronal cultures treated with HK or LK medium. The ability of the immunoprecipitated kinase to phosphorylate GST-IκB-β was analyzed using a construct containing residues $1–168^{S19A/S23A}$ of IκB-β and another construct in which Tyr-161 was mutated to Phe. As shown in Fig. 5, *A* and *B*, Arg phosphorylated IκB-β to a greater extent in HK. The elevated phosphorylation in HK was abolished when Tyr-161 was mutated. PD173955 is a highly selective inhibitor of c-Abl and Arg (27, 28). As shown in Fig. 5*A*, treatment of neuronal cultures with PD173955 blocks the ability of Arg to phosphorylate GST-IκB-β in HK medium. As seen with the GST-IκB-β construct, treatment of neuronal cultures with PD173955 also blocked HK-mediated phosphorylation of endogenous IκB-β (Fig. 6, *E* and *F*). However, phosphorylation of the GST-IκB-β proteins by c-Abl immunoprecipitants did not exhibit the differential pattern (Fig. 5, *C* and *D*). These results suggest that Arg is likely the kinase that mediates the differential phosphorylation of Tyr-161 in IκB-β. It has been reported that activity of c-Abl and Arg is tightly regulated by phosphorylation on tyrosine residues in these two kinases. Higher kinase activity of c-Abl/Arg correlates to higher tyrosine phosphorylation in these two proteins. We performed Western blot experiments to examine the tyrosine phosphorylation status of immunoprecipitated c-Abl and Arg in HK, LK, and HK with PD173955-treated neurons. HK treatment induces the tyrosine phosphorylation on Arg (Fig. 5*E*) and only slightly higher levels of tyrosine phosphorylation on c-Abl (Fig. 5*F*). PD173955 treatment abolishes the tyrosine phosphorylation on both Arg and c-Abl (Fig. 5, *E* and *F*), indicating that this drug does affect its targets *in vivo*. Moreover, the levels of immunoprecipitated Arg and c-Abl remain similar, regardless of the treatment, when the same blots were probed with Arg and c-Abl antibody, respectively. These data suggest that the regulation of Arg and

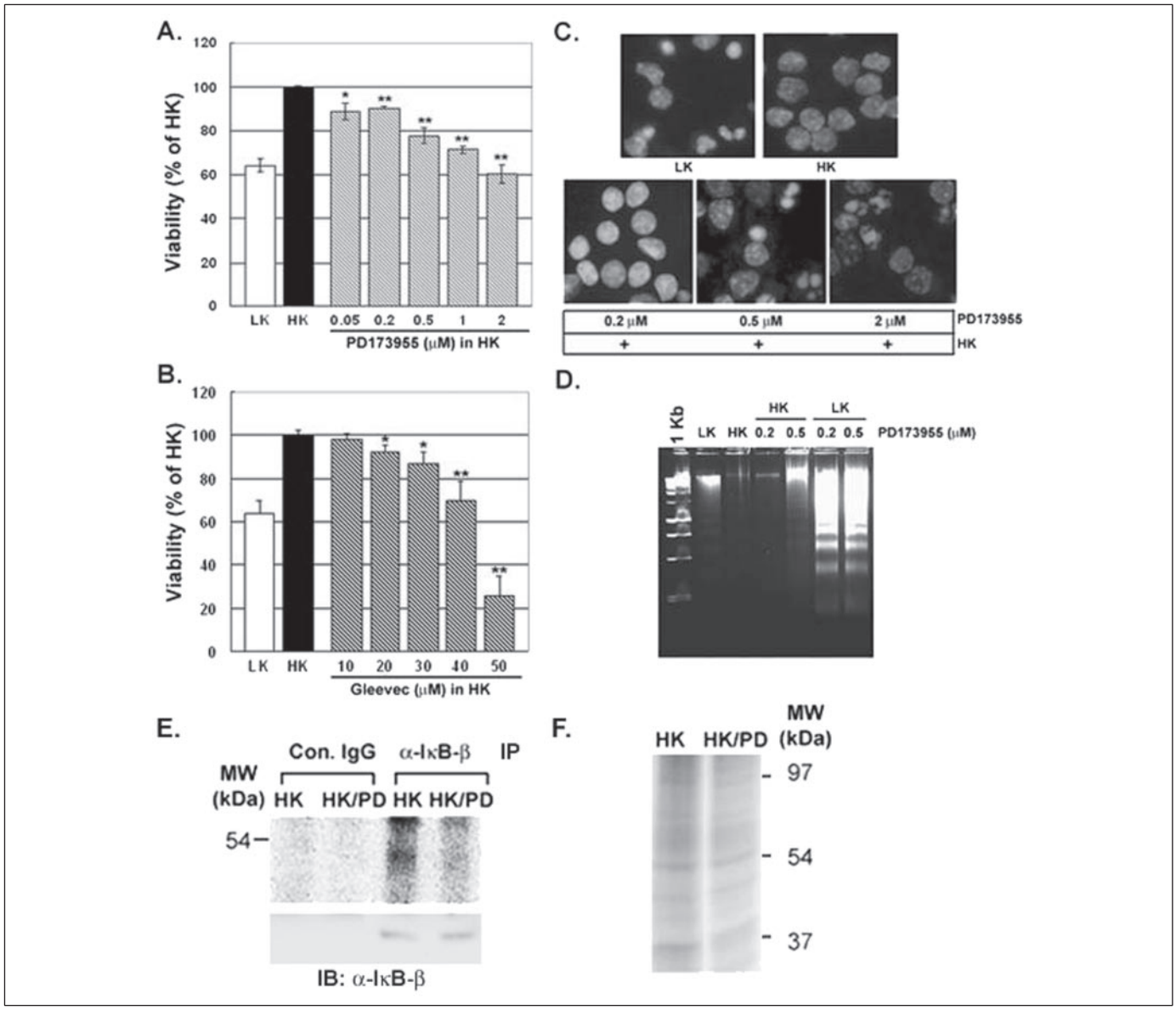

FIGURE 6. **Treatment with specific c-Abl inhibitors causes apoptosis and reduced IκB-β phosphorylation in HK-treated neurons.** Granular neurons were treated with various concentrations of c-Abl inhibitor PD173955 or Gleevec in HK. Viability of cells during the treatment was quantified by counting DAPI-stained cells. Survival rate for HK-treated neurons is set to 100. Values for other treatments were calculated based upon the relative level to the value of HK-treated neurons. Results from three separate experiments are analyzed and shown as mean ± S.E. *, $p < 0.05$; **, $p < 0.005$ on unpaired Student's *t* test when results from chemical treatment are compared with that of the HK treatment. *A*, viability of neurons with PD173955 treatment. *B*, viability of neurons with Gleevec treatment. *C*, DAPI staining images of neurons treated with PD173955. *D*, DNA fragmentation analysis was performed. The image of an agarose gel with DNA samples prepared from HK, LK, or HK with PD173955 and LK with PD173955-treated neurons. *E*, neurons were metabolically labeled with [^{32}P]orthophosphate and treated with HK or HK with PD173955 for 6 h. Lysates prepared from these neurons were immunoprecipitated with control IgG (*Con. IgG*) or IκB-β antibody. Immunoprecipitants (*IP*) were resolved on SDS-polyacrylamide gel followed by autoradiograph to PhosphorImager screen. Image for phosphor-IκB-β is shown (*top*). Same membrane was reprobed with IκB-β antibody (*bottom*), whereas the total tyrosine phosphorylation profile for the HK or HK/PD-treated neurons is shown in *F*. *IB*, immunoblot.

c-Abl activity under the conditions we used occurs at the activity level, and not because of a change in the protein level.

Abl Inhibitors Induce Apoptosis in HK Condition and Reduce IκB-β Phosphorylation in Vivo—The above results indicated that elevated IκB-β phosphorylation in HK happens at a site(s) not sensitive to IKK. We also showed that Tyr-161 of IκB-β is related to the induced phosphorylation and that Arg is the candidate kinase for this phosphorylation. We then conducted experiments to address the biological relevance of the above observations with regulating apoptosis in neurons. We treated neuronal cultures with PD173955, a highly selective inhibitor of Abl and Arg (27). As shown in Fig. 6*A*, treatment with PD173955 blocked HK-mediated survival in a dose-dependent manner. This result was confirmed using a second and structurally distinct inhibitor of Abl/Arg, Gleevec (29, 30), as shown in Fig. 6*B*. Arguing against a nonspecific toxic effect of these inhibitors is the finding that treatment with PD173955 caused nuclear condensation and the characteristically nonrandom fragmentation of DNA (Fig. 6, *C* and *D*). To confirm that the reduction in neuronal survival observed after pharmacological inhibition of Arg/Abl involved a reduction in IκB-β phosphorylation, we performed ^{32}P metabolic labeling experiments. IκB-β was immunoprecipitated from lysates of neuronal cultures that were labeled with ^{32}P and treated with PD173955. As shown in Fig. 6*E*, PD173955 treatment reduces phosphorylation but has no effect on protein level of IκB-β *in vivo*. The overall phosphorylation pattern in these cultures was similar, indicating that the reduction of IκB-β phosphorylation is specific (Fig. 6*F*).

Phosphorylated IκB-β Associates with p65 and Regulates NF-κB Binding Activity—In non-neuronal cell lines, IκB-β has been found to associate with p65 under some conditions causing an increase in NF-κB DNA binding activity (16). NF-κB activity in cerebellar granule neurons is higher in HK (2). Because the phosphorylation of IκB-β is also higher in HK, we examined whether IκB-β associated with p65 in neurons and, if so, what effect the phosphorylation of IκB-β had on its interaction with p65. Full-length GST-IκB-β-bound glutathione-agarose beads were incubated with cellular extracts prepared from neurons treated with HK or LK medium. Following the pull-down assay, p65 that associated with the exogenously added GST-IκB-β was then studied by Western blot by using a p65 antibody. As shown in Fig. 7*A*, association between GST-IκB-β and p65 was detectable under both HK and LK conditions. As observed with IκB-β phosphorylation, the extent of interaction was reduced in LK consistent with the possibility that association between p65 and IκB-β was regulated by the phosphorylation of IκB-β. To investigate this further, endogenous IκB-β was immunoprecipitated from ^{32}P-labeled HK- or LK-treated neuronal cultures, and the extent of p65 interaction was evaluated. As shown in Fig. 7*B*, both phosphorylation of IκB-β and its interaction with p65 was reduced in LK medium. The interaction between p65 and IκB-β was disrupted in the presence of excess amounts on an IκB-β peptide against which the antibody was made, but not by an unrelated peptide against the p35 protein.

To examine more directly the significance of IκB-β phosphorylation to its association with p65, we performed GST pull-down and *in vitro* kinase assay with nonradioactive ATP with lysates from HK- or LK-treated neuronal cultures. As shown in Fig. 7*C*, GST-IκB-β pulled down from HK-treated cultures was tyrosine-phosphorylated. The extent of tyrosine phosphorylation was greatly reduced when PD173955 was added to the *in vitro* kinase reaction. Not unexpectedly and consistent with what was observed in intact neurons, phosphorylation of GST-IκB-β was also reduced in LK-treated cultures. The amount of tyrosine phosphorylation correlated well with the amount of p65 that associated with GST-IκB-β. Thus, association between GST-IκB-β and p65 was clearly detectable when HK lysates were used but was barely detectable in the presence of PD173955, LK-treated lysates, or GST-IκB-βY161F. This mutant IκB-β displayed significantly lower tyrosine phosphorylation than wild-type IκB-β, and no interaction between p65 and IκB-β Y161F was observed.

To examine whether Abl/Arg-mediated phosphorylation of IκB-β affected the activity of NF-κB during neuronal apoptosis, we performed EMSA. As shown in Fig. 8*A* and as reported previously (2), the DNA binding activity of NF-κB activity is reduced in neurons primed to die by LK treatment. NF-κB binding activity is also reduced in HK with the presence of PD173955 (Fig. 8*B*). In contrast, neither LK nor PD173955 had a substantial effect on the DNA binding activity of Sp1 or TFIID. The DNA binding activity of NF-κB (Fig. 8*C*) was also reduced when the nuclear extracts were co-incubated with IκB-β or p65 antibody (Fig. 8*D*), suggesting the presence of these proteins in the complex. Taken together, these results suggest that IκB-β and NF-κB are associated in a DNA-binding complex that is stimulated by Arg-mediated phosphorylation of IκB-β.

Overexpression of Wild-type or Y161F Mutant of IκB-β Affects Neuronal Survival—To address further the *in vivo* function of IκB-β and the relationship of Tyr-161 phosphorylation to neuronal survival, we transiently expressed wild-type IκB-β or a mutant form of the protein in which Tyr-161 was mutated (IκB-βY161F) in cultured neurons. As shown in Fig. 9, forced expression of wild-type IκB-β protects neurons from LK-induced cell death. Consistent with the requirement for Arg-mediated phosphorylation, inclusion of PD173955 blocked protection by wild-type IκB-β. Further underscoring the importance of Arg-mediated IκB-β phosphorylation to neuronal survival is the finding that overexpression of IκB-βY161F decreases neuronal survival in both HK and LK conditions.

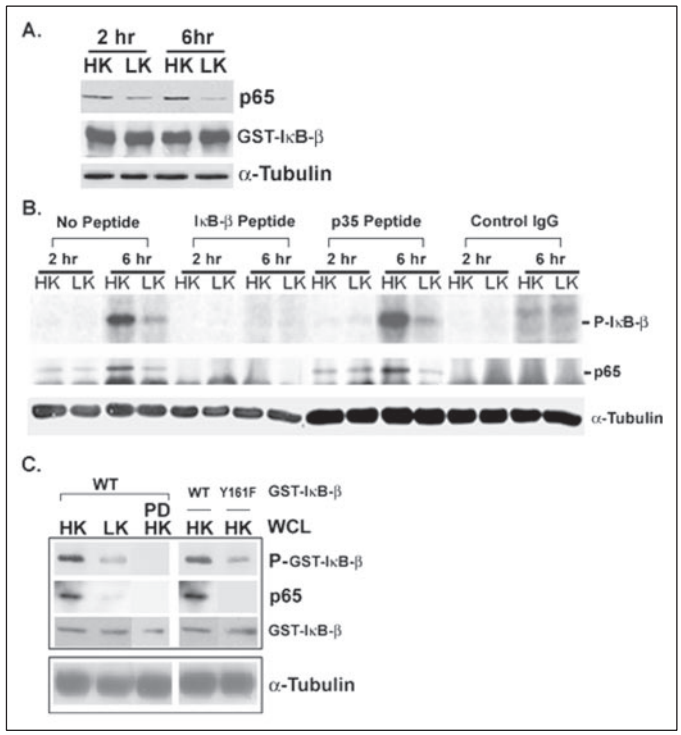

FIGURE 7. **Differential association of IκB-β-p65 in the HK- and LK-treated neurons and effect of Tyr-161 mutation and PD173955 on GST-IκB-β phosphorylation *in vitro*.** *A*, cellular extracts prepared from neurons with 2 and 6 h of treatment with HK and LK were applied to GST pull-down assay with GST-IκB-β-bound glutathione-agarose (full-length, wild-type). Western blot experiment was conducted with p65 antibody (*top*), GST antibody (*middle*), and α-tubulin (*bottom*) for input protein levels. *B*, neurons labeled metabolically with ^{32}P and treated with HK or LK for 2 or 6 h were lysed in RIPA buffer. Immunoprecipitation of whole cell lysates with IκB-β antibody was performed in the presence of specific or nonspecific competitive peptides. The autoradiograph is shown at the *top*. The same membrane was probed with p65 antibody (*middle*). Supernatants from the immunoprecipitation were subjected to Western blot with α-tubulin antibody (*bottom*). *C*, effect of tyrosine phosphorylation of IκB-β on its association with p65. Full-length GST-IκB-β (WT) or GST-IκB-β Y161F was bound to glutathione-agarose beads. GST pull-down and nonradioactive *in vitro* kinase assay in the presence of ATP (see "Experimental Procedures") was performed. Neurons were treated in HK or LK conditions for 6 h. Cells were lysed in RIPA buffer after treatment. *Upper panel,* PD-HK refers to a sample in which the pull down was performed from HK lysates, and 2 μM PD173955 added to the kinase reaction following the pull-down. The reaction samples on the beads were resolved on SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membrane. Western blotting was performed with phosphotyrosine (*1st row*), p65 (*2nd row*), or GST (*3rd row*) antibodies. *Lower panel,* Western blotting experiment was conducted for the flow-through samples with α-tubulin antibody. *WT*, wild type.

DISCUSSION

We report that IκB-β is phosphorylated in cerebellar granule neurons and that the level of phosphorylation is reduced in neurons primed to apoptosis by LK treatment. The higher phosphorylation of IκB-β in HK medium is not because of IKK. A mutant form of IκB-β with mutations in the two IKK-mediated phosphorylation sites and a C-terminally truncated form of IκB-β lacking the two phosphorylation sites still display differential phosphorylation in HK *versus* LK medium. We have mapped the apoptosis-regulated phosphorylation site to Tyr-161. A comparison of sequences from different mammalian species reveals that this is an evolutionarily conserved residue within IκB-β and hence one that may be important for the function of the protein. Indeed, over-

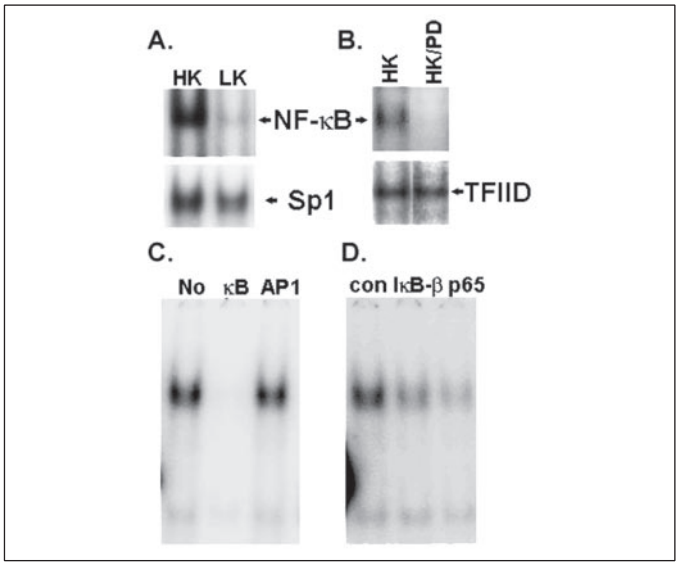

FIGURE 8. **Treatment with Abl/Arg inhibitor PD173955 reduces DNA binding activity of NF-κB in neurons.** Nuclear extracts prepared from cells treated with HK, LK, or HK+ PD173955 (2 μM) for 6 h are subjected to EMSA with various DNA-binding oligos. The types of treatment are indicated at the *top* of each panel. *A*, *upper panel*, DNA binding activity to κB oligos. *Lower panel*, DNA binding activity to Sp1 oligos. *B*, *upper panel*, DNA binding activity to κB-binding oligos. *Lower panel*, DNA binding activity to TFIID-binding oligos. *C*, nuclear extract from HK-treated neurons were subjected to EMSA to κB oligos. No (no competitive nucleotides), nonradioactive κB, and AP1 oligos were included in the assay for competition experiments. *D*, same nuclear extract as in *C* was incubated with control (*con*) IgG, IκB-β, and p65 antibody before addition of radiolabeled κB oligos for supershift experiment.

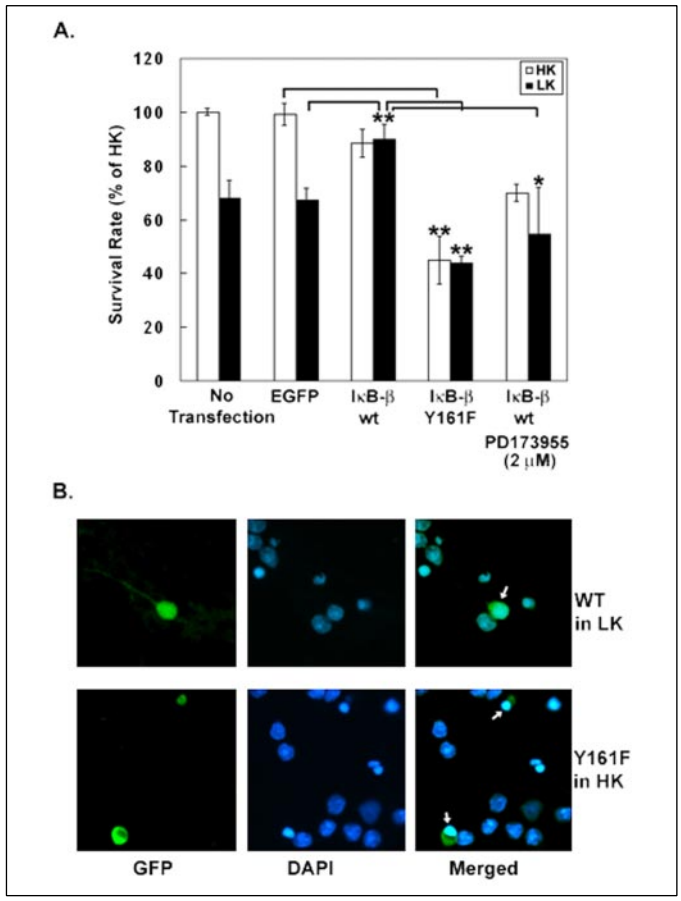

FIGURE 9. **Effects of IκB-β (wild-type and Y161F) on neuronal survival and impact of PD173955 on IκB-β-mediated neuroprotection *in vivo*.** Wild-type (*wt*) and Y161F mutant of full-length IκB-β were subcloned into pEGFP-N1 vector at the N terminus of EGFP. Transient transfection of cerebellar granular neurons was performed by using the calcium phosphate precipitation method. Cells were then treated in HK, LK, and HK with PD173955 (2 μM) conditions, respectively. DAPI staining was conducted after 24 h of treatment. Condition of transfected cells was visualized under fluorescence microscope with both UV and GFP filters, and the numbers were counted. *A*, statistical analysis for these transfection experiments is shown. Survival rate for HK-treated, nontransfected neurons is set to 100. Values for other treatments were calculated based upon the relative level to the value of HK-treated, nontransfected neurons. Results are derived from three separate experiments and shown as mean ± S.D. *, $p < 0.05$; **, $p < 0.005$ on unpaired Student's *t* test. *B*, representative images for the transfection experiments. GFP displays cells under GFP filter (*left panel*); DAPI stands for cells stained with DAPI and visualized under a UV filter (*middle panel*); *merged* refers to images that are generated from merging of both GFP and DAPI images (*right panel*). *Upper*, IκB-β (*WT*) transfected cell in LK condition. *Lower*, IκB-β (Y161F)-transfected cells in HK condition. *Arrows* point to transfected cells.

expression of a mutant form of IκB-β in which Tyr-161 is mutated to a nonphosphorylatable residue inhibits neuronal survival even in HK.

Tyr-161 bears the consensus sequence for phosphorylation by the Abl nonreceptor tyrosine kinases. The Abl family consists of c-Abl and its paralogue Arg. Both c-Abl and Arg are expressed in most neurons during development and in the adult brain with Arg being much more abundant than c-Abl in the adult brain (19). Although the functional significance of these Abl kinases in the brain are unclear, roles in neuronal migration, axonal guidance, and synaptic communication have been suggested (18, 20, 22, 31). In *Drosophila* deletion of the single Abl gene, d-*abl*, leads to severe central nervous system defects (21, 32). Although gross abnormalities have not been detected in $Abl^{-/-}$ or $Arg^{-/-}$ mutant mice (19), this is likely to be due to their overlapping roles. Double mutant $Abl^{-/-}Arg^{-/-}$ mouse embryos exhibit defects in neurulation, although the early death of these mice at embryonic day E10.5 precludes analysis of the roles of these proteins in brain development and maturation (19).

Consistent with their involvement in IκB-β phosphorylation, the activities of c-Abl and Arg are reduced when neurons are switched from HK to LK medium. Treatment of neurons with PD173955, a specific Abl inhibitor, reduced GST-IκB-β phosphorylation in HK-treated cultures to a level comparable with that observed in LK medium. Although both c-Abl and Arg interact with IκB-β, the differential pattern of endogenous IκB-β phosphorylation is recapitulated *in vitro* only by Arg implicating it in the phosphorylation of IκB-β. Underscoring the importance of Abl-mediated phosphorylation of IκB-β to neuronal survival is our finding that inhibition of this phosphorylation event using two distinct pharmacological inhibitors, PD173955 and Gleevec, leads to cell death.

How does the phosphorylation of IκB-β at Tyr-161 help neuronal survival? We find that IκB-β associates with p65, and association is enhanced by IκB-β. Mutation of Tyr-161, or the pharmacological inhibition of Abl kinases, reduces the association of IκB-β with NF-κB. The importance of NF-κB to neuronal survival has been well established in a number of neuronal paradigms (1, 2). In cerebellar granule neurons, the activity of NF-κB is reduced by LK treatment (2, 33). A similar reduction of NF-κB activity is observed when IκB-β phosphorylation is inhibited by PD173955 treatment. The crystal structure (Protein Data Bank codes IK3Z, crystal form I, and 1OY3 crystal form II) of a IκB-β-p65 complex reported recently reveals that this complex can bind to DNA (34). We have confirmed that IκB-β-bound p65 can bind DNA.[3] Studies in other laboratories have shown that IκB-β bound to p65 can enhance NF-κB DNA binding activity within the nucleus (15, 16). Taken together, these observations suggest that the phosphorylation of IκB-β increases NF-κB activity by stimulating DNA binding.

The best studied member of the IκB protein family is IκB-α. Although IκB-α and IκB-β have often been assumed to be interchange-

[3] L. Liu and R. D'Mello, unpublished observations.

able, a portion of IκB-β that is important for its subcellular localization is missing in IκB-α (34). Tyr-161 resides within this region. EMSA results from our laboratory using recombinant IκB-α shows that it inhibits the DNA binding of p65.[3] In cerebellar granule neurons and other neuronal cell types, overexpression of IκB-α inhibits NF-κB activity and induces apoptosis, whereas overexpression of either wild-type or a mutant form of IκB-β in which IKK-mediated phosphorylation sites are altered results in the survival of neuronal cells under apoptotic conditions.[3] Thus, in the context of neuronal survival, IκB-α and IκB-β have different effects.

In contrast to other members of the IκB family of proteins, mice lacking IκB-β have yet to be generated, and hence the physiological function of IκB-β is poorly understood. Our results implicate a novel role for IκB-β in the regulation of neuronal survival. This action of IκB-β is regulated by its phosphorylation by Abl kinases and leads to the activation of NF-κB. By understanding in more detail the molecular events leading to the inactivation of c-Abl and Arg in dying neurons and the mechanism by which reduced IκB-β phosphorylation ultimately leads to neuronal death may shed insight into the mechanisms underlying neurodegenerative diseases.

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